Evaluation of transgenic grapevine lines overexpressing Vv-AMP1 antifungal peptide

by

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Declaration

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Summary

The importance of small antimicrobial peptides in the innate immune system of plants became increasingly apparent over the past decade. Antimicrobial peptides are unique and diverse molecules that are found in many tissue types in a variety of invertebrate, plant and animal species. Many of these peptides, such as plant defensins, have been found to be ubiquitous throughout the plant kingdom and have been isolated from flowers, leaves, roots, seeds, seedlings, pods, tubers and bark.

The growing relevance of antimicrobial peptides (including plant defensins) in research can be largely attributed to their broad-spectrum antifungal activity. This makes them promising potential targets, both as therapeutic agents and for their use in crop protection and disease resistance. The continuing discovery of novel antimicrobial peptides has advanced the development of strategies to overexpress these genes in plants to attempt to enhance the plant's natural ability to resist pathogenic attack.

The first grapevine antifungal peptide, *Vv-AMP1*, was isolated and characterized and was shown to be tissue specific and developmentally regulated, being expressed only in berries at the onset of berry ripening. The peptide showed strong antifungal activity against a number of plant pathogenic fungi *in vitro*. In this study, the biological role of the *Vv-AMP1* peptide was further investigated, both within its native host (*Vitis vinifera*) and under *in vitro* conditions against a panel of grapevine-specific pathogens.

As a first step, recombinant production of Vv-AMP1 using an existing bacterial expression system was evaluated and the heterologous production of the Vv-AMP1 peptide improved. Specific optimizations targeting both production and purification of the peptide showed to improve the yield of Vv-AMP1. Steps in the production process targeted for improvement included induction conditions of peptide production by the bacterial culture as well as a number of purification steps, such as lysate preparation, binding conditions, column washing, elution conditions and thrombin protease cleavage. The optimized purification method produced up to 3 mg of pure Vv-AMP1 peptide from 1.6 L of overnight culture. While production was markedly improved, the resultant purified Vv-AMP1 proved biologically inactive and structurally unstable. This is uncharacteristic of the peptide, suggesting that an important aspect necessary for peptide activity, such as folding or the presence of specific co-factors might not be supported in this non-host prokaryotic production system.

The study also entailed the characterization and evaluation of the Vv-AMP1 peptide against a panel of grapevine-specific pathogens that are culturable to

sporulating cultures using *in vitro* antifungal assays and microscopy analysis. Vv-AMP1 showed strong inhibitory activity against all pathogens tested, inhibiting the growth of *Diplodia seriata* and *Cylindrocarpon liriodendri* by 50% at concentrations between 4.8 µg/ml and 9.6 µg/ml. *Phaemoniella chlamydospora* and *Phomopsis viticola* proved particularly sensitive, with IC_{50} values of 5.5 µg/ml and 4.0 µg/ml respectively. Microscopy analysis of the effect of the Vv-AMP1 peptide on *P. viticola* showed a severe inhibition on fungal germination and growth. The peptide did not induce morphological changes in fungal hyphae but compromises the fungal membranes, supporting the theory that the peptide induces membrane permeabilization.

Functional analysis of a transgenic *V. vinifera* (cv. Sultana) population overexpressing *Vv-AMP1* was included in this study to provide the opportunity to study the *in planta* role of the peptide in its native host. The genetic characterization of the putative population included confirming gene integration and copy number through PCR and Southern blot analysis as well as gene expression through northern blot analysis. A confirmed transgenic population was evaluated for improved disease resistance against *Botrytis cinerea* as a first test organism in an attempt to link the overexpression of the *Vv-AMP1* gene to a disease resistance phenotype. Observations of lesion type, average lesion size and further statistical analysis concluded that the transgenic population showed a definite, albeit slight, improved resistance when compared to the untransformed control lines.

In conclusion, the study determined that Vv-AMP1 had a strong antifungal action against grapevine-specific pathogenic fungi when tested *in vitro*. A definite link could be established between the overexpression of Vv-AMP1 and a mild resistance phenotype within its native host plant. The characterized transgenic population is important for further work to evaluate the *in planta* activity of the peptide against more grapevine pathogens such as the stem pathogens that were proven sensitive and specifically those that cannot be cultured and are obligate pathogens, such as the downy and powdery mildews.

Opsomming

Die belang van klein antimikrobiese peptiede in die ingebore immuunstelsel van plante het tydens die afgelope dekade toenemend duidelik geraak. Antimikrobiese peptide is unieke en diverse molekules wat in verskeie weefseltipes in ‘n verskeidenheid van invertebraat-, plant- en dierspesies gevind word. Baie van hierdie peptiede, soos bv. “plant defensins”, word bevind om alomteenwoordig in die plantryk te wees en is reeds geïsoleer vanuit blomme, blare, wortels, sade, saailinge, peule, knolle en bas.

Die toenemende belang van antimikrobiese peptiede (insluitend “plant defensins”) in navorsing kan grootliks toegeskryf word aan hul breë-spektrum antifungiese aktiwiteit. Hierdie eienskap maak hul belowende potensiële teikens, beide as terapeutiese middels asook vir gebruik in gewasbeskerming en siekteweerstand. Die voortdurende ontdekking van nuwe antimikrobiese peptiede bevorder tans die ontwikkeling van strategieë om hierdie gene in plante uit te druk in ‘n poging om die plant se natuurlike vermoeë om patogeniese aanval teen te staan te verbeter.

Die eerste wingerd antifungale peptied, *Vv-AMP1*, is geïsoleer en gekarakteriseer as ‘n ontwikkelings-gereguleerde peptied wat slegs uitgedruk word in korrels, tydens die aanvang van bessie rypwording. Die peptied het tydens *in vitro* toetse sterk antifungale aktiwiteit getoon teen ‘n verskeidenheid plant-patogeniese swamme. In hierdie studie word die biologiese rol van die Vv-AMP1 peptied verder ondersoek, beide binne sy natuurlike gasheerplant, (*Vitis vinifera*) asook onder *in vitro* kondisies teen ‘n paneel van wingerd-spesifieke patogene.

As ‘n beginpunt is rekombinante produksie van Vv-AMP1 met behulp van ‘n bakteriële ekspressie sisteem evalueer en die hetereloë produksie van die Vv-AMP1 peptied stelselmatig verbeter. Spesifieke optimerings het gefokus op beide die produksie en suiwering van die peptied en het die algehele opbrengs van Vv-AMP1 verhoog. Spesifieke stappe wat in die produksieproses vir verbetering geteiken is sluit beide induksietoestande van peptiedproduksie deur die bakteriële kultuur in sowel as ‘n aantal suiweringsstappe, soos lisaatvoorbereiding, bindingskondisies, kolom wasstappe, eluasie kondisies en “thrombin” protease snyding in. Die optimale suiweringsmetode het tot 3 mg suiwer Vv-AMP1 peptied opgelewer vanaf ‘n 1.6 L oornag bakteriële kultuur. Hoewel die produksie van die peptide noemenswaardig verbeter is, was die gesuiwerde Vv-AMP1 beide onaktief en struktureel onstabiel. Dit is buitengewoon vir hierdie peptied, wat daarop dui dat belangrike aspekte benodig vir

antifungiese aktiwiteit, soos korrekte vou of die teenwoordigheid van spesifieke ko-faktore, moontlik ontbreek in hierdie nie-gasheer prokariotiese produksiesisteem.

Die studie het ook die karakterisering en evaluering van die Vv-AMP1 peptied teen 'n paneel van wingerd-spesifieke patogene wat kultureerbaar is en sporuleer, insluitend *in vitro* antifungale toetse en mikroskopiese analise, behels. Vv-AMP1 toon sterk inhiberende aktiwiteit teen alle patogene getoets. Dit inhibeer die groei van *Diplodia seriata* en *Cylindrocarpon liriodendri* met 50% teen konsentrasies tussen 4.8 µg/ml en 9.6 µg/ml. *Phaemoniella chlamydospora* en *Phomopsis viticola* was besonders sensitief, met IC_{50} waardes van 5.5 µg/ml en 4.0 µg/ml, onderskeidelik. Mikroskopiese analise van die effek van die Vv-AMP1 peptied op *P. viticola* het 'n ernstige inhibisie op swam ontkieming en groei aangedui. Die peptied het geen morfologiese veranderinge in swam hifes veroorsaak nie maar het wel die swam membraan beskadig. Hierdie bevinding ondersteun die teorie dat die peptied membraan permeabilisasie induseer.

Funksionele analise van ‘n transgeniese *V. vinifera* (cv. Sultana) populasie wat die *Vv-AMP1* geen ooruitdruk is by die studie ingesluit om ‘n geleentheid te bied om die *in* planta rol van die peptide binne sy natuurlike gasheerplant te bestudeer. Die genetiese karakterisering van die vermeende transgeniese bevolking het die bevestiging van beide geenintegrasie en kopiegetal deur PKR en Southern-klad analise ingesluit, sowel as geenuitdrukking d.m.v. noordelike-klad analise. ‘n Bevestigde transgeniese bevolking is evalueer vir potensiële verbeterde weerstand (in vergelyking met die wilde tipe) deur infeksie met *Botrytis cinerea* as ‘n eerste toetsorganisme in ‘n poging om ‘n weerstandbiedende fenotipe met die ooruitdrukking van *Vv-AMP1* te assosieer. Waarnemings van letsel tipe, letsel grootte en verdere statistiese analise het tot die gevolgtrekking gelei dat die transgeniese bevolking ‘n definitiewe (dog geringe) verbeterde weerstand toon in vergelyking met die ongetransformeerde lyne.

Ten slotte bepaal die studie dat Vv-AMP1 ‘n sterk antifungale effek teen wingerd-spesifieke patogene toon tydens *in vitro* toetse. ‘n Definitiewe korrelasie is vasgestel tussen die ooruitdrukking van Vv-AMP1 in wingerd en ‘n weerstandsfenotipe in die transgeniese bevolking. Die gekarakteriseerde transgeniese bevolking is uiteraard belangrik vir toekomstige werk om die *in planta* aktiwiteit van die peptied te evalueer teen verdere wingerdpatogene soos bv. die stampatogene wat sensitief getoets het teen die peptide, asook patogene wat nie kultureerbaar is nie, insluitend verpligte patogene soos dons- en poeierskimmel.

This thesis is dedicated to
Hierdie tesis is opgedra aan

My ouers
My parents

Biographical sketch

Marthèlize Tredoux was born in Bellville, South Africa, on 2 December 1984, and was raised in Strand. She matriculated from Strand High in 2002 and commenced her studies at the University of Stellenbosch in 2003 where she enrolled for a BSc degree in Animal Biotechnology. After graduating in 2005, she pursued post-graduate study, obtaining a BScHons degree in Wine Biotechnology in 2006 and starting her MSc degree in Wine Biotechnology in 2007. She enrolled for her LLB degree at the University of South Africa in January 2011.

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Preface

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the journal Plant Physiology.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
Antifungal Peptides: A Review

Chapter 3 **Research results**
Heterologous production of Vv-AMP1, a defensin from grapevine, in *Escherichia coli*

Chapter 4 **Research results**
Vv-AMP1, a defensin from grapevine, shows strong antifungal activity *in vitro* and overexpression in grapevine slightly improves *in planta* resistance against *Botrytis cinerea*

Chapter 5 **General discussion and conclusion**

I hereby declare that I was the primary contributor with respect to the experimental data presented on the multi-author manuscripts presented in Chapter 3 and 4. My supervisors were involved in the conceptual development and continuous critical evaluation of the study. Dr K Vasanth transformed the grapevine population that was analysed in this study and reported in Chapter 4.

Contents

Chapter 1

General introduction and project aims

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Since the onset of organized agriculture, plant disease caused by viruses, fungi and bacteria have affected crops of all types, causing major losses a negative effect on crop quality. At present, agricultural disease control is mainly anchored in the use of chemicals to lessen the damage (Shah, 1997; Agrios, 2005). Modern agriculture also applies crop rotation as means of crop protection, but despite all these measures many pathogens prove formidable adversaries. Moreover, many pathogens are developing resistance to certain fungicides and some treatments are proving increasingly ineffective to decrease the occurrence of disease (Staub, 1991).

While the development of new and improved methods of crop protection are essential, the focus of these methods must also be to comply with the safety concerns considering the potential negative impact many of these chemicals have on human health and the environment (Colosio et al., 2008). In addition to safety concerns, methods must also prove to be cost-effective. Spraying with chemicals is a costly process, and often the treatment must be repeated to be effective (Staub, 1991; Ma and Michailides, 2005).

This situation is especially relevant to grapevine, the world's most important and widely cultivated fruit crop (Vivier and Pretorius, 2002). Worldwide, the majority of commercially important grapes are constituted by different cultivars of *Vitis vinifera*. The rest are other species of *Vitis* or interspecific hybrids. Seventy percent of grapes produced are used for wine, 22% for table grapes and 8% for raisins. There are also a number of by-products or derivatives of the wine industry that have economic importance, such as must, marc distillates, marc pulp, tartaric acid, seed oil and vinegar (Troggio et al., 2008). *V. vinifera* is susceptible to fungal pathogens, carrying no innate resistance to mildew fungi such as powdery (*Uncinula necator*) and downy mildew (*Plasmapora viticola*), and also to microbial and viral attack (Figueiredo et al., 2008). This necessitates the use of regular and intense spraying of phytochemicals, at great costs, both monetary and environmentally. These negative aspects drive research into alternative methods to enhance the plant's innate defense system (Le Henanff et al., 2009).

With the commencement of the molecular era of plant biology in the early 1980's, a large part of research has been dedicated to genetically engineer crops for improved disease resistance (Day, 1986; Tuzun et al., 2006). During the last 30 years, technology surrounding plant transformation and regeneration in general has improved dramatically (Vidal et al., 2009; Prado et al., 2010). Grapevine is a particularly difficult plant to transform, with established protocols involving complex procedures requiring specialized expertise. Even though many of these protocols are established, most have low transformation efficiency (Reustle and Buchholz, 2009). Methods for genetic transformation of grapevine have progressed and been developed extensively since the first successful transformations in 1989 (Baribault et al., 1989). Many research groups dedicate their work to improving the efficiency of transformation protocols and the improvement of transformation procedures. Improved, detailed protocols for both *Agrobacterium*-mediated transformation (Bouquet et al., 2006) and biolistic bombardment (Kikkert et al., 2005) have been published (Reustle and Buchholz, 2009).

In addition to focusing on the improvement of grapevine transformation methods, most transgenic grapevine research aims to improve physiological traits but also establish resistance against viruses, fungi and bacteria (Reustle and Buchholz, 2009).

Identifying, cloning and characterizing genes involved in disease resistance has been improving significantly in recent years (Hollaender et al., 1985; Tuzun et al., 2006). These genes are often targeted as transgenes for the genetic improvement of crops due to their potential to improve the plant's ability to defend itself against pathogens. Plant defensins are small, basic antimicrobial peptides that form part of the innate immune system. They are functionally related to other defensins found in insects and mammals and seem to be ubiquitous throughout the plant kingdom, having been described in a number of diverse plant species (Carvalho and Gomes, 2009). Plant defensins exhibit a broad range antifungal action but are nontoxic to mammalian and plant cells. They are produced through transcription and translation of a single gene, which means their delivery after infection is rapid and with relatively low input of biomass and energy (Thomma et al., 2002).

Through advances in molecular screening techniques, the discovery of plant defensins has been increasing exponentially in recent years. It has been proposed that the number of defensin-like genes in plants is greatly under-predicted (Graham

et al., 2004; Silverstein et al., 2005). In recent years, this has proven to be an accurate postulation. The difficulty in accurately predicting these genes is linked to the extreme sequence diversity within these gene families, with most molecular techniques only being able to identify closely related sequences (Graham et al., 2008). With the development of genome-scale sequencing technology, whole-genome analysis is now more readily available to researchers. In addition, the availability of fully sequenced plant genomes allows whole genome analysis using expressed sequence tag (EST) libraries. Many agriculturally important crops such as *Oryza sativa* (rice), *Sorghum bicolour* (sorghum), *Zea mays* (corn), *Malus domestica* (apple) and *V. vinifera* (grapevine) have fully sequenced genomes, enabling more targeted research strategies.

These advances in genome analysis have led to the re-evaluation of previous findings related to defensin-like genes in plants. Graham et al. (2004) showed that plant genomes typically have hundreds of defensin-like genes. In the *Arabidopsis* genome alone, more than 300 defensin-like peptides have been identified, 78% of which possess the characteristic cysteine-stabilized α-helix β-sheet (CSαβ) motif, common in all plant and invertebrate defensins (Thomma et al., 2002; Silverstein et al., 2005). This is in severe contrast to the 15 defensins previously described in *Arabidopsis* (Thomma et al., 2002). Considering the size of the gene families, it suggests that our functional knowledge of defensins is still rather limited.

The continuing discovery of novel plant defensins has advanced the development of strategies to overexpress these genes in plants in an attempt to enhance the plant's innate immunity and consequently improve resistance against pathogen attack. In designing disease resistant crops, a resistant characteristic of a donor organism is transferred to the desired crop through recombinant DNA technology. A well-publicized example of crops engineered for pest resistance is cotton and maize, engineered to express and produce the Bt toxin (from *Bacillus thuringiensis*), which is poisonous to insect pests. While the crops produce the toxin in their tissue, the effects are targeted specifically to lepidopteron pests, purportedly with no effect on any other organisms in the surrounding soil, including earthworms, nematodes, protozoa, bacteria and fungi (Saxena and Stotzky, 2001).

Despite pervasive controversy surrounding transgenic crops and genetic engineering, both industrial and developing countries continue to plant more hectares of transgenic crops year after year. In 2009, 77% of the world's soybean

crop was transgenic; 49% of cotton, 26% of maize and 21% of canola were all transgenic. Across the globe, transgenic crops made up 134 million hectares, which is an 80-fold increase from 1996 (Figure 1). This translates to a yearly growth of 9 million hectares or 7%. In South Africa alone, the estimated total biotech crop was 2.1 million hectares, which is mainly made up of soybean, maize and cotton. It has also been suggested that planting transgenic crops can lead to a reduction in CO_2 emissions resulting from fewer insecticide and herbicide sprays (James, 2009).

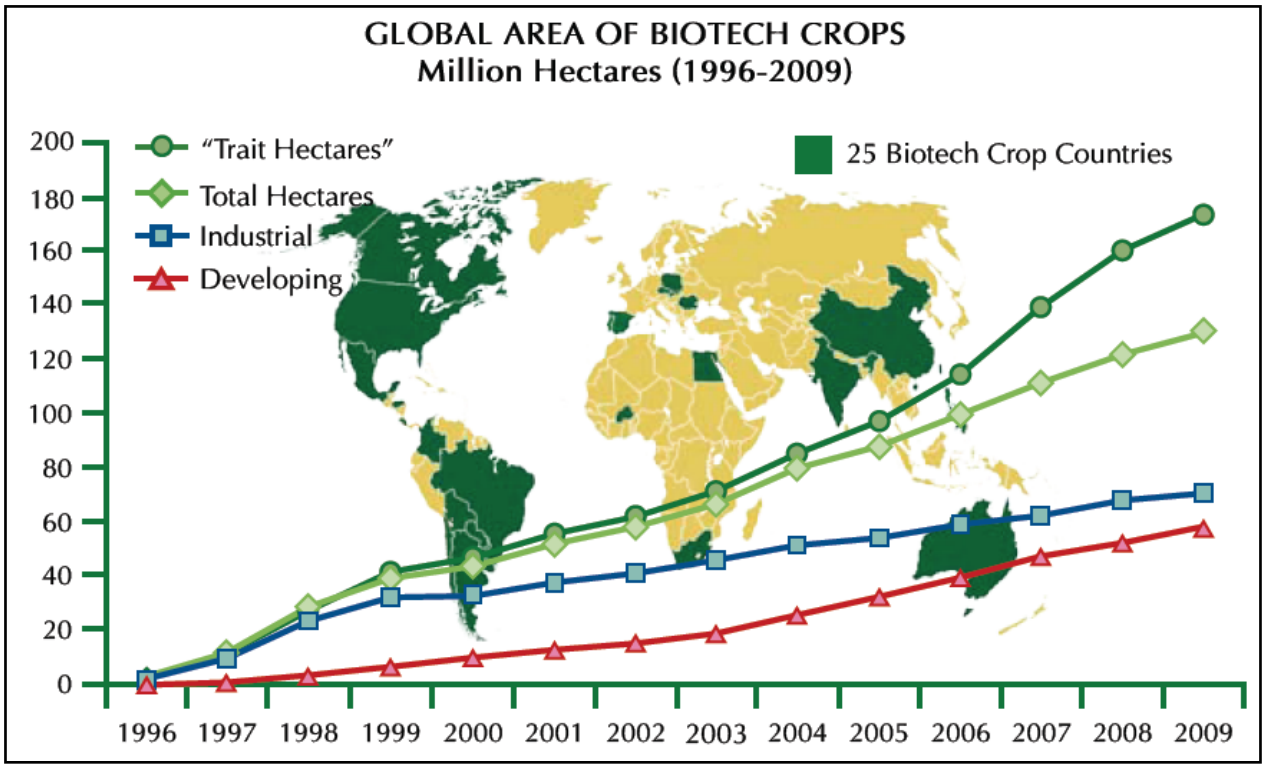

Figure 1. Representation of the growth of transgenic or "biotech" crops between 1996 and 2009. The map indicates countries, both industrial and developing, that use transgenic crops commercially. The term "trait hectare" refers to an area of transgenic crop containing stacked traits, and is calculated by multiplying the surface area with the number of GM traits in the crops. It is therefore not an indication of actual surface area but rather "virtual" hectares (James, 2009).

While public opinion often mistrusts genetically modified crops, the benefits the technology offers seem to far outweigh the possible risks. Not only do some genetically engineered crops show improved resistance against disease, thereby lessening the need for harmful chemical treatments, but the possibility of an associated decrease in carbon emissions and the promise of food security for

developing countries more than confirms the need to further expand and improve the field.

The use of transgenic technology is a powerful tool in many research areas, including agriculture and pharmacology. The ability to transfer genes and gene products between organisms or to alter expression of a native gene in a host organism allows the opportunity to further investigate the function and effects of the gene within the system. This study will continue the functional characterization of an antifungal peptide isolated from grapevine. Previously, the peptide was isolated and characterized *in vitro* and overexpressed in tobacco as an infection model plant (De Beer, 2008). This work will be extended by evaluating the activity against grapevine pathogens and overexpressing the peptide in its native host, grapevine.

1.2 PROJECT AIMS

The first grapevine defensin from *V. vinifera* was designated *Vv-AMP1* (*Vitis vinifera* antimicrobial peptide 1) and was isolated and characterized by De Beer and Vivier (2008). The peptide encoding gene showed developmentally regulated, tissue specific expression, only being expressed in berries and at the onset of berry ripening and onwards. Expression of the *Vv-AMP1* gene could not be induced by hormone treatment, wounding or infection. Further analyses revealed that the signal peptide allowed accumulation of the peptide in the apoplastic region. Recombinantly produced Vv-AMP1 had a molecular mass of 5.495 kDa, as determined by mass spectrometry. The peptide was extremely heat stable and showed strong antifungal activity against a range of plant pathogenic fungi. Vv-AMP1 was tested against *Botrytis cinerea*, *Fusarium solani*, *F. oxysporum* and *Verticillium dahliae* and inhibited 50% of fungal growth at concentrations of 13, 9.6, 6 and 1.8 µg/ml respectively. Although the peptide did have a damaging effect on fungal membranes, it did not induce morphological changes such as hyperbranching and was classified as non-morphogenic (De Beer and Vivier, 2008).

Overexpression in tobacco and subsequent challenging of the population with *B. cinerea* did not lead to a resistance phenotype in a detached leaf assay. Although expression of the transgenes were confirmed, it was not possible to detect the presence of Vv-AMP1 in the plant with Western blot, due to the presence of native peptides in tobacco that cross-reacted with the Vv-AMP1 polyclonal antibody (De

Beer, 2008). Questions remained regarding the *in vivo* function, activity and stability of the peptide, some of which will be pursued in this study.

This project was initiated to further explore the biological role of the peptide when overexpressed within its native host as well as to evaluate the *in vitro* activity of the peptide against a panel of grapevine-specific pathogens. This required the recombinant production of pure Vv-AMP1 peptide for *in vitro* antifungal assays (according to a method by Broekaert et al., 1990) to determine the level of activity of the peptide against a panel of suitable grapevine-specific pathogens.

A transgenic population of *V. vinifera* cv. Sultana transformed with a Vv-AMP1 overexpression cassette was obtained from the Institute for Wine Biotechnology (IWBT) grapevine transformation and regeneration platform. This population will be phenotypically and genetically analyzed to evaluate the functional role of Vv-AMP1 in grapevine.

The specific aims of the project were as follows:

a. To recombinantly produce and evaluate purified Vv-AMP1 peptide
    i. Evaluation and optimization of a bacterial expression system
    ii. Heterologous production and purification of Vv-AMP1 peptide
b. To evaluate and characterize the antifungal activity of the Vv-AMP1 peptide against a panel of grapevine specific pathogens
    i. *In vitro* antifungal assays to determine the specific IC_{50} values of the peptide against each pathogen
    ii. Microscopic analysis of the inhibition of Vv-AMP1 on the different pathogens to evaluate the mode of action
c. The analysis of transgenic *V. vinifera* (cv. Sultana) lines overexpressing the Vv-AMP1 peptide
    i. Multiplication and maintenance of putatively transgenic lines to form a mother and working collection of *in vitro* and hardened off grapevine lines. Observation of the lines for any obvious visible phenotypes linked to the overexpression of the peptide
    ii. Genetic analysis of a putative transgenic population of *V. vinifera* transformed with *Vv-AMP1* and the untransformed controls

- PCR and Southern blot analysis to determine gene integration and copy number
- Northern blot analysis to determine gene expression
- Assays to confirm peptide production and activity

iii. Infection studies of the confirmed transgenic population and controls to evaluate potential resistance phenotype linked to the overexpression of the *Vv*-AMP1 gene in grapevine

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Chapter 2

Literature review

Antifungal peptides – A Review

LITERATURE REVIEW

2.1 Introduction

All living organisms are continuously exposed to potentially harmful microbes and pathogens. Despite this constant threat of infection, the occurrence of disease is the exception rather than the rule. The ability of multi-cellular organisms to withstand disease depends on their capacity to actively defend themselves against pathogen attack. As a result, all forms of life (including microbes) have evolved mechanisms of host defense, involving varied components and responses (Broekaert et al., 1997; Reddy et al., 2004; Linde et al., 2009).

All types of organisms have to defend themselves against pathogens using one (or both) of two immune systems: nonspecific immunity or specific immunity. Nonspecific immunity is also referred to as innate immunity, pre-formed immunity, rapid immunity or simply as the host defense system. In all multicellular organisms, this type of immunity serves as the first line of defense against pathogen attack. Innate immunity utilizes a number of antimicrobial substances, ranging from inorganic chemicals (e.g. hydrogen peroxide, hypochlorous acid, nitric oxide) to enzymes (e.g. proteases, muramidases) and other proteins and peptides with antimicrobial action to prevent or restrict the ability of the invading microbes to establish infection (Ganz and Lehrer, 1995; Raj and Dentino, 2002; Nicolas and Rosenstein, 2009).

Mechanisms of innate immunity are genetically predetermined and require no previous exposure to the specific pathogen (Boman, 1995; Izadpanah and Gallo, 2005). Endogenous peptides involved in host defense are typically constitutively expressed, although some have been shown to be inducible. Effector molecules of innate immunity (whether chemical, enzymatic or peptides) are generally produced by the transcription and translation of a single gene. This allows rapid delivery of the gene product with very limited energy expense (Thomma et al., 2002). This non-specific branch of host defense is conserved throughout both plant and animal kingdoms (including invertebrates and fungi) indicating its ancient origins (Brown and Hancock, 2006; Linde et al., 2009).

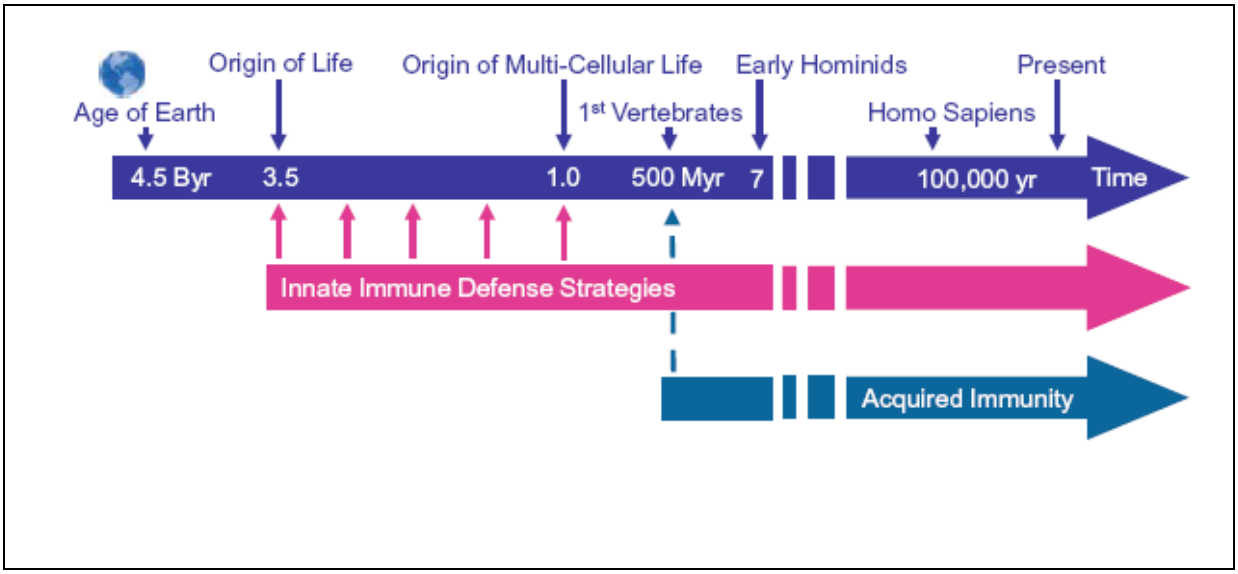


Figure 2.1 Evolution of immune defense systems. Innate immunity is generally considered the evolutionary most ancient of the two main branches of immunological defense systems dating back to the first multi-cellular organisms or earlier (note change in time scale – *Byr* billion years, *Myr* million years) (Adopted from Linde et al., 2009).

Specific immunity (also known as adaptive or acquired immunity) is found exclusively in mammals. Compared to innate immunity, acquired immunity is a more recently evolved system of defense, found only in higher vertebrates (Figure 1). Acquired immunity is more complex than its ancient counterpart. It is both specific and has a memory function, enabling the organism to “remember” and recognize a specific pathogen through antigen recognition by antibodies (Izadpanah and Gallo, 2005), facilitating it to deal more efficiently with subsequent challenges from the same organism (Broekaert et al., 1997; Linde et al., 2009). It does not rely on gene-encoded products, but rather the activation of T and B cells against specific antigens (Reddy et al., 2004).

Owing to the need for the host organism to first recognize the pathogen, the acquired immune response is slightly delayed. With microbes having a very short doubling time (some as quick as 20 minutes), this delay could prove detrimental to the host in allowing the pathogen enough time to establish infection. The rapid action of the innate immune response provides the host with almost immediate protection, without requiring activation of the adaptive immunity (Marshall and Arenas, 2003).

Consequently, the importance of the innate immune response (or host defense) in preventing disease becomes clear, both in organisms with and without adaptive immune systems. Antifungal peptides form part of a large group of antimicrobial

peptides (AMPs). The former are significant role-players in the host defense system of both plants and animals and will form the subject of this review, with a specific focus on plant defensins.

2.2 Classification of Antimicrobial peptides (AMPs)

Antimicrobial peptides are diverse and unique molecules found in many tissue and cell types of a variety of invertebrate, plant and animal species (Figure 2). On account of their role as first line of defense against infection, they are most abundant in cells, tissues and organs that are constantly exposed to microbes (Schröder, 1999). In animals they are prevalent in the cutaneous tissues, mucous membranes, respiratory tract lining fluids, skin, pancreas, kidney, salivary glands, prostate and endocervix (Raj and Dentino, 2002; Benko-Iseppon et al., 2010). In plants they have been isolated from flowers, leaves, seeds, seedlings, pods, tubers, roots and bark (Schröder, 1999; Lay and Anderson, 2005). Insect antimicrobial peptides are typically found in the haemolymph, the functional equivalent to blood (Otvos, 2000). Avian defense peptides are distributed similarly to those of mammals and have been isolated from epithelial cells; heterophils; peripheral leukocytes; the respiratory, digestive and urogenital tract and the skin (van Dijk et al., 2008).

While the diversity and distribution of AMPs in the Eukaryotic domain is evident (see Figure 2), the different peptides (although dissimilar in details of their structure and function) all share a fundamental structural principle in their amphipathic design. Different classes of peptide achieve this through differing structural characteristics (e.g. cecropins and magainins assume an amphipathic α-helical structure when entering a membrane, while defensins possess a rigid anti-parallel β-sheet, stabilized by disulphide bonds) (Zasloff, 2002).

AMP primary sequences are so diverse that the same sequence is rarely isolated from two different species of animal, even if they are closely related. There is, however, considerable conservation of amino acid sequences, even between different classes of peptides, different species and even across kingdoms (Zasloff, 2002).

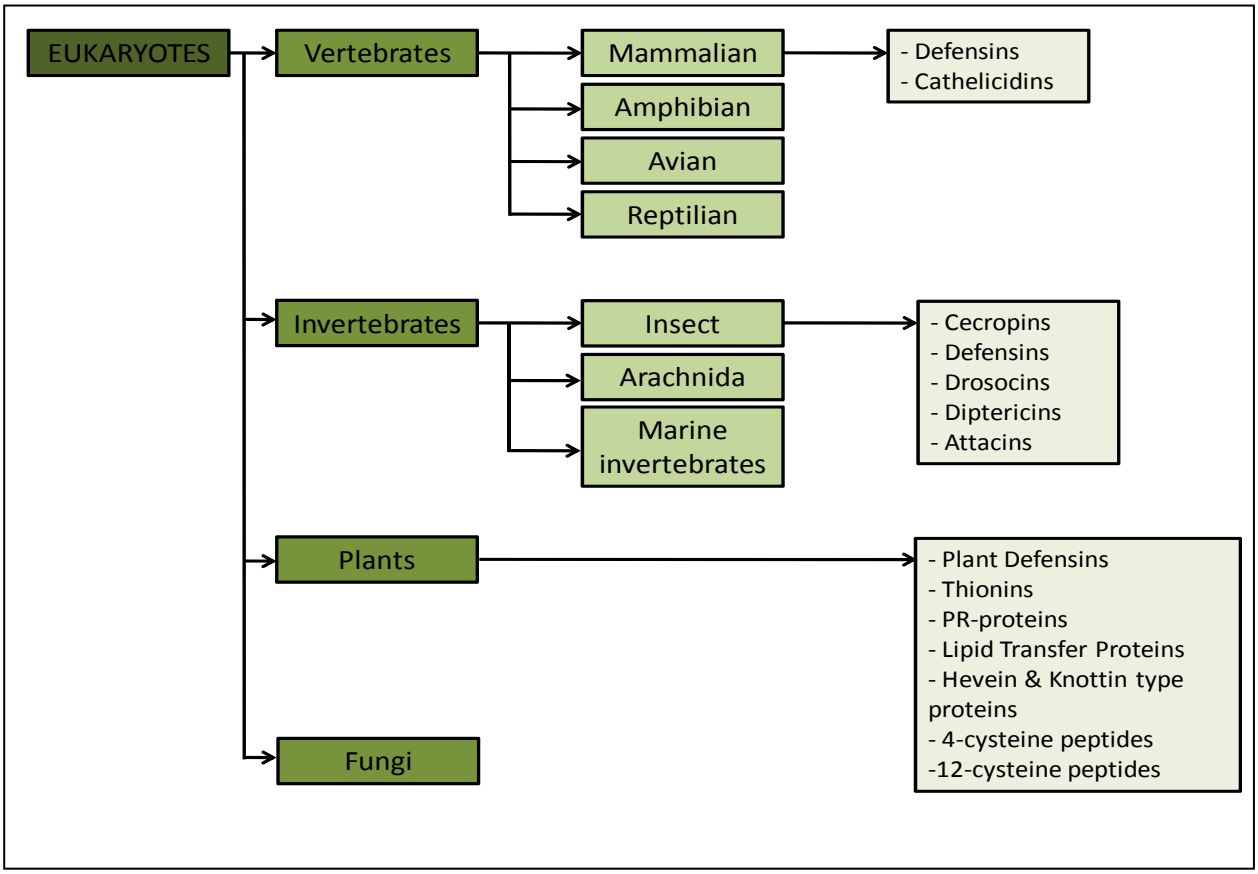


Figure 2.2 Distribution of antimicrobial peptides across the Eukaryote domain. AMPs are arranged according to kingdom, class and type of AMP (Compiled from Ganz and Lehrer, 1995; Garcia-Olmedo et al., 1998; Bulet et al., 1999; Zasloff, 2002; Castro, 2005; van Dijk et al., 2008; Linde et al., 2009; Otero-Gonzalez et al., 2010).

AMPs are generally classified into subgroups based on their size, conformational structure and predominant amino acid composition, as summarized in Table 1 (Zasloff, 2002; Marshall and Arenas, 2003). It has been noted that the AMPs are so diverse that categorization in one generally accepted classification is rather difficult (Koczulla and Bals, 2003).

Table 2.1 Classes of antimicrobial peptides according to amino acid composition and structure (adapted from Vizioli and Salzet, 2002; Zasloff, 2002).

Structure	Representative peptides	Source organism	Reference
Anionic peptides	Maximin H5	Toad	(Lai et al., 2002; Diamond et al., 2009)
	Dermcidin	Human	(Schittek et al., 2001; Lai et al., 2007)
Linear α-helical peptides	Cecropins	Insects	(Bulet et al., 1999; Coca et al., 2006; Jin et al., 2010)
	Magainin	Amphibians	(Andreu and Rivas, 1998; Gregory et al., 2009)
	Buforins	Amphibians	(Park et al., 1996; Conlon, 2004; Hao et al., 2009)
Linear peptides rich in certain amino acids	Proline-rich: - drosocin	Fruit fly	(Bulet et al., 1999; Bikker et al., 2006)
	Glycine-rich: - diptericins	Dipterans	(Bulet et al., 1999; Johansson et al., 2006)
	Histidine-rich: - histatin	Human	(Andreu and Rivas, 1998; Smet and Contreras, 2005)
	Tryptophan-rich: - indolicidin	Cattle	(Andreu and Rivas, 1998; Rokitskaya et al., 2010)
Single disulfide bridges	Brevinins	Frog	(Andreu and Rivas, 1998; Basir et al., 2000; Conlon et al., 2009)
Two disulfide bridges	Protegrin	Pig	(Kokryakov et al., 1993; Bolintineanu et al., 2010)
	Tachyplesin	Horseshoe crab	(Dimarcq et al., 1998; Cirioni et al., 2007)
	Androctonin	Scorpion	(Dimarcq et al., 1998)
Three disulfide bridges	α-defensins	Mammals	(Ganz and Lehrer, 1995; Hazrati et al., 2006)
	β-defensins	Mammals	(Ganz and Lehrer, 1995; Bullard et al., 2008)
	Insect defensins	Insects	(Dimarcq et al., 1998; Bulet et al., 1999; Aerts et al., 2008)
	Penaeidins	Shrimp	(Destoumieux-Garzòn et al., 2001; Ho and Song, 2009)
Four or more disulfide bridges	Drosomycin	Fruit fly	(Fehlbaum et al., 1994; Zhang and Zhu, 2009)
	Plant defensins	Plants	(Fehlbaum et al., 1994; García-Olmedo et al., 1998; Benko-Iseppon et al., 2010)

2.3 AMP research – History and Relevance

The correlation between microbes and disease dates back to the early 19th century. Robert Koch, Louis Pasteur and their contemporaries were the first to suggest the notion that microbes were the causal agents of food spoilage and disease. The work of Joseph Lister and Paul Ehlrich focused on the search for antimicrobial substances that could be used to combat microbial infection (Ganz, 2005).

In 1929, Alexander Fleming identified lysozyme as the first enzyme showing antimicrobial properties. He also indicated that different forms of lysozyme were widely distributed throughout the plant and animal kingdoms (Fleming, 1922). In 1932, his discovery of penicillin effectively sparked the beginning of antimicrobial research (Fleming, 1932; 1944).

Throughout the 20th century, a number of antibiotic and antimicrobial substances have been discovered and isolated from insects (Hultmark et al., 1980), plants (García-Olmedo et al., 2001) and animals (Hirsch, 1956; Zeya and Spitznagel, 1968). Towards the end of the century, improvements in molecular biology led to the conclusion that AMPs are encoded by gene families. It allowed the purification of individual peptides, the determination of their amino acid sequences and the cloning of the genes that encoded these peptides. By the mid-1990's research on AMPs extended to invertebrates, vertebrates, plants and bacteria. In 1994, researchers from the various fields of AMP research met for the first time at a CIBA symposium in London (Ganz, 2005). Collaboration and interaction between the various research groups propelled the field of antimicrobial research forward, and publications on AMPs in recent years have increased significantly (Figure 3).

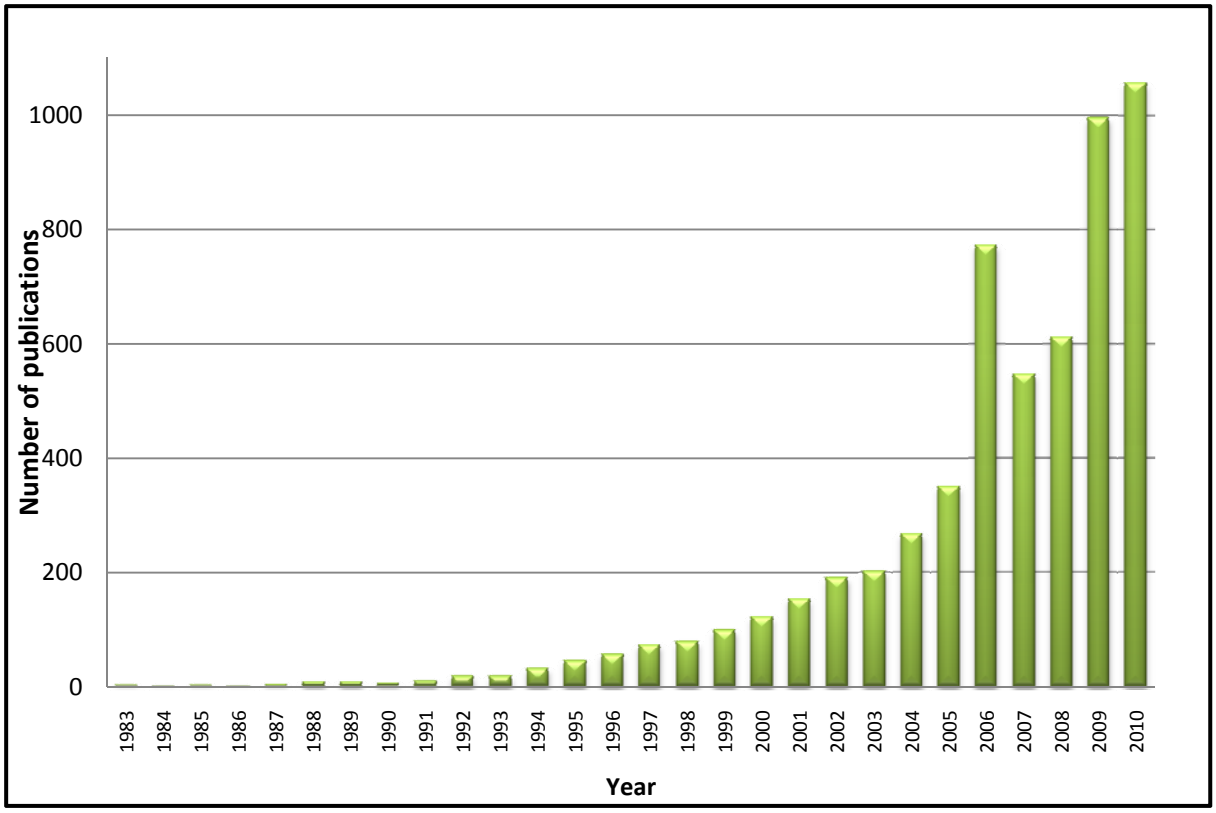


Figure 2.3 Publications on antimicrobial peptides. Relevant publications were identified by a web-based search in PubMed using the search term “antimicrobial peptide” (adapted from Koczulla and Bals, 2003).

2.3.1 Growing importance of AMP research

It is evident that antimicrobial peptide research has increased dramatically over the past decade and specifically in the last five years (Figure 3). This can largely be attributed to the recent recognition of the economic importance of these peptides. Their broad-spectrum activities make them suitable targets for development as therapeutic agents and for their potential use in crop protection and disease resistance (Shah, 1997; Reddy et al., 2004).

2.3.2 AMPs as promising therapeutics

Since the industrial-scale production of penicillin started in 1943, the availability of antibiotics has had a profound effect on human life. It has contributed to an eight-year increase in the average human lifespan and has allowed the successful treatment of bacterial infections which previously would have been injurious or even fatal (Hancock, 1998).

Throughout the years however, bacterial strains have gradually adapted to become increasingly resistant to conventional antibiotics. For example, 95% of all *Staphylococcus aureus* strains tested are resistant to penicillin (Breithaupt, 1999). This alarming rise in antibiotic resistance (including the emergence of untreatable infections from multi-drug resistant strains of *Mycobacterium tuberculosis* and *Enterococcus*) highlights the need for novel antimicrobial agents (Hancock and Patrzykat, 2002). AMPs possess many desirable features as novel antimicrobial compounds: they possess a broad spectrum of activity, they kill bacteria rapidly and some show no toxicity towards eukaryotic cells (Hancock and Scott, 2000; Wilcox, 2004). Nonetheless, there are still issues surrounding the use of AMPs as antibiotics that need to be solved, not least of which is cost-effective production. While there are several methods established for recombinant production of peptides, none have yet successfully been applied on an industrial scale (Hancock and Scott, 2000).

Aside from their potential as antibiotics, AMPs also show potential as antiviral (acting against enveloped viruses such as hepatitis and HIV (Nakashima et al., 1992), anticancer (some peptides actively attack cancer cells) (Moore et al., 1994); wound healing (Hancock, 1998) agents and even as contraceptives (Reddy et al., 2004).

To date, very few antimicrobial peptides have been entered into clinical trials, with varied success. One of the longest running and most successful trials to date is from Micrologix Biotech Inc. (now known as Migenix Inc., Vancouver, British Columbia, Canada). In 1999, they entered an indolicidin-like peptide, named MBI-226 or Omiganan™, into Phase I (safety) trials. In 2000 they received fast-track status from the Food and Drug Administration. In 2002 they initiated two more clinical trials using indolicidin-like peptide, against acute acne and against methicillin-resistant *S. aureus* (MRSA) (Hancock and Patrzykat, 2002; Portieles et al., 2006).

To date, Omiganan™ has been evaluated for two topical applications, for the treatment of catheter-related infections as well as the treatment of dermatological diseases. Two Phase III clinical trials have been completed for the treatment of catheter-related infections. The results did not meet the endpoint of the study and further development of the drug is being investigated. Two Phase II clinical trials have also been completed for Omiganan™ in the treatment of rosacea and acne. Enrollment in Phase III trials is currently pending (Migenix, 2009).

2.3.3 Biotechnological application of AMPs

Management and prevention of disease in food crops has become a global industry, dedicated to developing new and improved methods of preventing and controlling the incidence and spread of disease. Factors that negatively affect crops include disease, pests and weeds. These competing factors influence both the quality and quantity of crop production (Walker, 1983). In agriculture, disease caused by pathogens has become increasingly difficult to manage.

Current agricultural disease control is based on the spraying of chemicals (herbicides, pesticides and fungicides) to regulate and reduce the occurrence of disease-causing organisms. Spraying, however, is not a once-off treatment and must be regularly repeated to have a lasting effect. This is a costly process, and with many pathogens developing resistance to commonly used chemicals (Staub, 1991; Ma and Michailides, 2005), even regular treatment can prove ineffective to lessen the incidence of disease.

The excessive use of chemicals is becoming less desirable, not only because of the financial implications, but also owing to environmental concerns and potential negative impact on human health and safety. Studies have linked exposure to pesticides to diseases (Colosio et al., 2008) such as Parkinson's disease (Ascherio et al., 2006), cancer (Landau-Ossondo et al., 2009), birth defects (Winchester et al., 2009) and more directly, acute pesticide poisoning (Van Der Hoek et al., 1998).

In light of these concerns, research has been focused on increasing the disease resistance of crops against pathogen attack through alternative approaches that are less harmful to both humans and the environment. The most recent and notable approach is genetically engineering crops for resistance (Shah, 1997; Gao et al., 2000; Kanzaki et al., 2002; Jacobsen et al., 2009).

Overexpressing genes encoding for antifungal peptides into crops may possibly enhance the plant's natural ability to defend itself against infection. The active production of these antifungal peptides by the plant could provide the plant with the ability to inhibit fungal growth and slow down infection. The abundance of antifungal peptides that have been discovered to date provides a wide range to choose from when designing a transgenic strategy.

Table 2.2 Plant defensins overexpressed in transgenic hosts (adapted from Lay et al., 2005)

Transgene	Source organism	Recipient plant(s)	Test organism(s) to evaluate resistance phenotypes	Reference
Rs-AFP2	Radish	Tobacco	*Alternaria longipes*	(Terras et al., 1995)
AlfAFP	Alfalfa	Potato	*Verticillium dahliae*	(Gao et al., 2000)
Spi1	Norway spruce	Tobacco, Norway spruce embryonic cultures	*Erwinia carotovora, Heterobasidion annosum*	(Elfstrand et al., 2001)
BSD1	Chinese cabbage	Tobacco	*Phytophthora parasitica*	(Park et al., 2002)
hBD-2	Human	*Arabidopsis thaliana*	*B. cinerea*	(Aerts et al., 2007)
Vv-AMP1	Grapevine	Tobacco	*B. cinerea*	(De Beer, 2008)
Dm-AMP1	*Dahlia merckii*	Rice	*Magnaporthe oryzae, Rhizoctonia solani*	(Sanjay et al., 2009)
Chili defensin	*Capsicuum annum*	Tomato	*Fusarium* sp., *Phytophthora infestans*	(Zainal et al., 2009)

Some of the best known and characterized plant AMPs are defensins. Plant defensins have been extensively targeted for enhanced resistance studies. Table 2 lists a number of studies overexpressing plant defensins in transgenic hosts. These

studies are often carried out either in model systems (such as tobacco) or in economically important crops (such as rice or potato). In one of the first studies of its kind, the constitutive expression of radish defensin (Rs-AFP2) in tobacco enhanced the plant's resistance to *Alternaria longipes* (Terras et al., 1995). An even more successful example of the ability of defensins to confer disease resistance to transgenic crops was the constitutive expression of alfAFP in potato. Not only did the transgenic lines show a six-fold decrease in the levels of *Verticillium dahliae* when compared to the non-transgenic plants, but this resistance was maintained under glasshouse conditions, field conditions and for several years thereafter (Gao et al., 2000). More recently, the Dm-AMP1 defensin was overexpressed in rice and showed resistance against both *Magnaporthe oryzae and R. solani*. The expression of the peptide in the apoplastic regions of the tissue may cause it to interact directly with fungal membranes, leading to membrane destabilization and in doing so, imparting enhanced disease resistance against a broad range of fungal pathogens to the transgenic plants (Sanjay et al., 2009). Another promising study was the overexpression of a chili defensin gene in tomato. The resultant transgenic lines were more resistant to both *Fusarium* sp. and *Phytophthora infestans*, though further research is required to determine whether this approach would be an effective means of increasing disease resistance (Zainal et al., 2009).

2.4 Concise overview of antifungal peptides in plants

Antimicrobial peptides play a key role in plant defense against invading pathogens. They form part of pre-existing defense barriers and are also components of the defense response induced upon infection. Generally, a peptide is classified as antimicrobial if "it interferes with the growth, differentiation, multiplication and/or spread of microbial organisms". Plant AMPs are classified into protein families based on homology, amino acid sequence and three-dimensional folding pattern (Broekaert et al., 2000).

2.4.1 Classification of plant antimicrobial peptides

Plant-derived AMPs are diverse and possess a broad range of biological activity, including antibacterial (Zhang and Lewis, 1997), insecticidal (Chen et al., 2002), protein synthesis inhibition (Harrison et al., 1997), α-amylase inhibition (Bloch and Richardson, 1991) and anti-HIV (Wong, 2005; Wong and Ng, 2005). Yet, it is their antifungal activity that appears to be ubiquitous throughout all plant species. Selitrennikoff (2001) categorized antifungal proteins and peptides, based on their mechanisms of action, structure or similarity to other proteins (Table 3). Accordingly, the remainder of this review will focus mainly on antifungal plant peptides, specifically plant defensins.

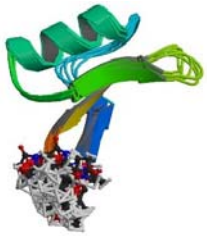
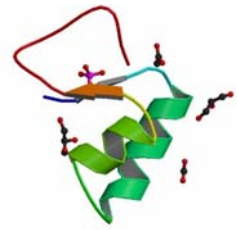
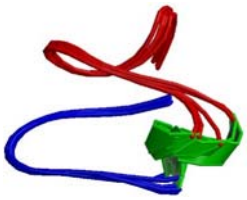
2.4.2 Antifungal peptides in plants

Of the nearly countless plant antimicrobial proteins isolated to date, a large proportion share common characteristics. They are typically highly basic proteins of small molecular weight (<10 kDa) with an even number of cysteine residues (typically 4, 6 or 8) that stabilize the protein structure through formation of disulfide bridges and provide structural and thermodynamic stability to the protein (Hancock and Lehrer, 1998; Lay and Anderson, 2005; Benko-Iseppon et al., 2010). Bearing in mind the similarities between different types of peptides, a number of distinct families have been identified. They include plant defensins (Broekaert et al., 1995; Broekaert et al., 1997; Lay et al., 2003), thionins (Bohlmann et al., 1994; Florack and Stiekema, 1994), lipid transfer proteins (Kader, 1996), hevein-type proteins (Broekaert et al., 1992), knottin-type proteins (Cammue et al., 1992), cyclotides (Craik et al., 1999), four-cysteine peptides (e.g. Ib-AMPs from *Impatiens balsamina* seeds) (Lee et al., 1999) and twelve-cysteine peptides (e.g. snakins) (Segura et al., 1999; Berrocal-Lobo et al., 2002) (see Table 4 for a comparison of these families, as well as an indication of numbers of amino acids and disulfide bridges, as well as the protein structure).

Table 2.3 Classification of antifungal proteins (Selitrennikoff, 2001), co-reported in (Ferreira et al., 2007). The defensins are boxed in this classification.

Class	Source	Characteristics	Mechanism of Action
PR-1 Proteins	Plants	Molecular masses of 15-17 kDa Homology to cysteine-rich proteins	Unknown
β-glucanases	Microorganisms, Plants Vertebrates, Invertebrates	1,3-β-Endoglucanase activity	Hydrolysis of the structural 1,3-β-glucan present in the fungal cell wall
Chitinases	Viruses, Bacteria, Fungi, Snails, Fish, Plants, Insects, Mammals, Amphibians	Chitinase activity Molecular masses of 26-43 kDa	Cleavage of cell wall chitin polymers
Chitin-binding proteins	Bacteria, Plants, Insects, Crustaceans	Chitin-binding proteins Molecular masses of 13-14.5 kDa	Binding to cell wall β-chitin (mechanism not understood)
Thaumatin-like proteins	Plants	Share significant sequence homology to thaumatin Molecular masses ~22 kDa	Precise mechanism not completely understood. Fungal cell permeability changes. Binding to 1,3-β-glucan. 1,3-β-glucanase activity
Defensins	Mammals, Fungi, Insects, Plants	Low-molecular mass, cysteine-rich peptides	Mechanisms not clearly elucidated. Acts on fungal membranes, leading to ion efflux
Cyclophilin-like proteins	Bacteria, Plants, Animals, Fungi	Intracellular receptors for cyclosporin	Unknown
Ribosome-inactivating proteins (RIPs)	Fungi, Plants	RNA *N*-glycosidases that depurinate RNA	Inactivates fungal ribosomes
Lipid Transfer Proteins (LTPs)	Mammals, Plants, Fungi, Bacteria	Molecular masses of ~8.7 kDa	Unknown
Protease Inhibitors	Plans, Animals, Microorganisms	Protein inhibitors of serine and cystein protease	Unknown
Other proteins	Plants	E.g. Viridin, Snakin-1	Unknown

Table 2.4 Small, cysteine-rich antimicrobial peptides from plants (Lay and Anderson, 2005; Benko-Iseppon et al., 2010). AA denotes the number of amino acid residues and DB the number of disulfide bridges.

Family	Representative member	AA	DB	Protein structure	Reference
Plant defensin	Rs-AFP1	51	4		(Terras et al., 1995)
Thionin	α-purothionin	45	4		(Ohtani et al., 1977)
Lipid Transfer Protein	Ace-AMP1	93	4		(Cammue et al., 1995)
Hevein-type	Ac-AMP2	30	4		(Broekaert et al., 1992)
Knottin-type	Mj-AMP1	36	3		(Cammue et al., 1992)
Cyclotide	Kalata B1	29	3		(Jennings et al., 2001)
Four-cysteine	Ib-AMP1	20	-	-	(Patel et al., 1998)
Twelve-cysteine	Snakin-1	63	-	-	(Segura et al., 1999)

Perhaps the most important of these families in plants, are the plant defensins. The first members of the plant defensin family were isolated from wheat and barley grains (Colilla et al., 1990; Mendez et al., 1990). They were originally classified as a new subgroup of thionins and named “γ-thionins” on account of their amino acid sequence similarity to α- and β-thionins. Further investigation on this new class of thionin revealed that they, in truth, showed a low degree of structural similarity to thionins and it was established that thionins and γ-thionins were unrelated (Terras et al., 1992; Bruix et al., 1995). In 1995, Terras et al. proposed the term “plant defensin” be used to describe these peptides and their homologs in plants (Terras et al., 1995).

The term was again used in the same year by Broekaert et al. (1995) in a review article which suggested that plant defensins share more structural similarity with defensins of vertebrate and invertebrate origin than with thionins. This significant homology (which included the occurrence of eight Cys residues, two Gly residues, an aromatic residue and a Glu residue at positions resembling analogous conserved residues in plant defensins) suggests that plant defensins belong to a superfamily of antimicrobial peptides, with representatives in vertebrates, invertebrates and plants, indicating that these defense molecules predate the evolutionary divergence of animals and plants (Broekaert et al., 1995; Lay and Anderson, 2005; Benko-Iseppon et al., 2010).

2.4.3 Mode of antifungal action of plant defensins

Plant antifungal peptides have been shown to inhibit the growth of a broad range of phytopathogenic fungi and even in some cases human fungal pathogens (e.g. *Candida albicans*) at very low concentrations (Broekaert et al., 1997; Thevissen et al., 2004). Very few plant defensins seem to possess antibacterial activity, although there are exceptions, e.g. fabatin-1 and -2 (Zhang and Lewis, 1997).

Osborn et al. (1995) observed that different types of plant defensins exert different physiological effects when tested against *Fusarium culmorum*. Based on these observations, two groups of defensins can be distinguished. “Morphogenic” plant defensins are characterized by reducing hyphal elongation with a concurrent increase in hyphal branching in treated hyphae. “Non-morphogenic” plant defensins also reduced the rate of hyphal elongation, but do not bring about any marked morphological changes (Osborn et al., 1995). It has to be noted that this morphogenic/non-morphogenic effect of defensins can be dependent on both the test fungus and test medium and is therefore not an absolute means to classify defensins.

The exact mechanism of antifungal action, whether it be morphogenic or not, has to date not been elucidated (Broekaert et al., 2000; Lay and Anderson, 2005; Portieles et al., 2006). A number of common observations regarding possible modes of action have been made. It has, for instance, been noticed that the antifungal activity of plant defensins against a test fungus is markedly reduced in the presence of monovalent and divalent cations in the growth medium (Broekaert et al., 1992; Osborn et al., 1995).

It was also noted that the antifungal activity was slightly more reduced by Ca^{2+} than by Mg^{+}. The same phenomenon has been observed for other small, basic, antimicrobial proteins, including insect and mammalian defensins (Cociancich et al., 1993; Bals et al., 1998). It is generally accepted that plant defensins act at the level of the plasma membrane of the fungus, as seems to be implied by the rapid Ca^{2+} influx and K^{+} efflux witnessed when radish (Rs-AFP2) and dahlia (Dm-AMP1) defensins are added at inhibitory concentrations to the hyphae of the fungus *Neurospora crassa* (Thevissen et al., 1996; 1999).

Fungi grow from the tip, which requires the maintenance of an intracellular Ca^{2+} concentration to drive polarized growth (Garrill et al., 1993; De Samblanx et al., 1997). Growth inhibition caused by plant defensins may be a result of the dissipation of this gradient as a result of ion flux caused by the peptide (Thevissen et al., 1996). The clear link between ion flux and antifungal activity was illustrated by De Samblanx and colleagues. A variant of Rs-AFP2 with enhanced antifungal activity caused an increased uptake of Ca^{2+}, while a second variant with no antifungal activity caused no Ca^{2+} uptake (De Samblanx et al., 1997).

Despite evidence hinting at possible modes of action, for most plant defensins, the molecular components involved in signaling and putative intracellular targets remain unknown. Only recently have research groups been able to reveal part of the molecular basis for antifungal activity in some plant defensins (Aerts et al., 2008).

Dm-AMP1 (from dahlia), Rs-AFP2 (from radish) and Hs-AFP1 (from coral bells) were the first plant defensins to provide clues to their mode of antifungal action. Dm-AMP1 and Rs-AFP1 inhibit fungal and yeast growth by inducing a range of rapid responses in fungal cells, including increased K^+ efflux and Ca^{2+} influx, alkalinization of the medium and membrane potential changes (Thevissen et al., 1996). Membrane permeabilization by the peptides was only detected at levels around 10 times more than the concentration inhibiting 100% growth. It was also evident that membrane permeabilization only occurred 2-4 hours after initial addition of the peptides to the hyphae. This suggests that permeabilization of membranes by plant defensins is a secondary effect of their antifungal activity, rather than the cause of the observed ion-flux (Thevissen, 1999).

Radiolabeled Dm-AMP1 was used to demonstrate the existence of high-affinity binding sites on fungal cells and membrane fractions. The binding site for Dm-AMP1 was identified as mannosyldiinositolphosphoryl-ceramide [$M(IP)_2C$]. Yeast mutants deficient in the $M(IP)_2C$ biosynthesis genes (*IPT1* and *SKN1*) proved resistant to Dm-AMP1. ELISA-based binding studies also indicated that Dm-AMP1 interacts directly with *Saccharomyces cerevisiae* sphingolipids (Thevissen et al., 2000; 2003; 2005).

Similarly, it was revealed that yeast mutants deficient in the glucosylceramide (GlcCer) biosynthesis gene *GCS1*, are resistant to Rs-AFP2 (Thevissen et al., 2004). This occurrence lends itself to explain the inherent resistance of *S. cerevisiae* and *C. glabrata* to Rs-AFP1, since they naturally lack GlcCer in their membranes. Through ELISA-based binding assays, it was shown that while Rs-AFP2 interacts with GlcCer isolated from *Pichia pistoris*, it fails to react with GlcCer from soybean or human membranes (Thevissen et al., 2004). This seems to account for non-toxicity of Rs-AFP1 to plant and human cells.

MsDef1 (previously named AlfAFP) is a broad-spectrum antifungal defensin from alfalfa (*Medicago sativa*) seeds. MsDef1 inhibits fungal elongation of *Fusarium graminearum* whilst causing a hyperbranching phenotype (Spelbrink et al., 2004). It is known that hyphal elongation is controlled by a gradient of cytosolic Ca^{2+}. Disruption of this gradient is known to cause hyperbranching (Tsien and Tsien, 1990). These observations gave rise to the hypothesis that MsDef1 blocks Ca^{2+} channels, thereby causing hyphal growth defects. The suggestion that certain antifungal peptides inhibit fungal growth through the blocking of Ca^{2+} channels is supported by studies demonstrating that fungal elongation is a complex process which relies heavily upon a maintained calcium gradient (Shaw and Hoch, 2001).

A more recent theory is that plant antifungal peptides exert their antimicrobial activity not only through membrane-related actions, but also through cytoplasmic targets (Xiong et al., 1999; Aerts et al., 2008; López-García et al., 2010). Psd1 defensin (from *Pisum sativum*) increased the DNA content of conidial cells without subsequent completion of cell-division. This proposes that Psd1 affects the normal progression of the cell cycle (Lobo et al., 2007).

Recent studies have therefore suggested that the mode of action of antifungal peptides extend beyond cell lysis and membrane permeabilization and that additional mechanisms are at play (Amien et al., 2010; López-García et al., 2010). The role of the cell wall in AMP mode of action is also being investigated. López-García et al. (2010) tested two unrelated AMPs against *S. cerevisiae* to examine the antifungal effect of the peptides. They showed that a weakened cell wall does not necessarily lead to a higher sensitivity to an AMP (López-García et al., 2010) suggesting that the cell wall itself may have a role in the AMP mode of action. Similarly, Amien et al. (2010) showed that permeabilization of fungal hyphae by the plant defensin NaD1 also involves a process that may be cell wall-dependent. Fungi with compromised cell walls were treated with NaD1, but the peptide showed a decrease in potency. This indicates that the peptide may interact with a proteinaceous receptor found on the cell wall itself.

2.4.4 Developmental role of antimicrobial peptides

Antimicrobial peptides are clearly an integral part of innate immunity in plants, insects and mammals. Most studies focus on the characterization of the *in vitro* antimicrobial activity of these peptides, or deciphering their modes of action. A third, non-defense role of these peptides is becoming clear. It has been suggested that many of these peptides have a definite role in developmental regulation of the tissues they are native to. This phenomenon has been observed in both mammalian and plant peptides.

A β-defensin named Bin1b is a rat epididymis-specific peptide (with a homologue found in humans). Bin1b is both structurally and with regards to antimicrobial activity similar to β-defensins. The region-specific expression of this peptide led researchers to investigate a possible role in sperm maturation. It was concluded that not only does Bin1b kill bacteria, it also plays a role in the maturation of sperm in the epididymis (Zhou et al., 2004).

DEF2, a defensin isolated from tomato, shows strong antifungal activity against *Botrytis cinerea*. The peptide is expressed during early flower development, and initial sense and antisense expression studies seem to indicate the peptide has a role in development and pollen viability (Stotz et al., 2009).

Allen et al. (2008) investigated the effects of plant defensins MsDef1, MtDef2, RsAFP2 and fungal toxin KP4 on plant root growth. They showed that all four peptides block root growth in a reversible manner, but that the inhibitory activity on the plant root was not directly correlated with the antifungal activity of the peptides. They suggest that these plant defensins have regulatory roles in plant development and growth (Allen et al., 2008). In maize, a cysteine-rich defensin-like peptide (ZmES4) was shown to induce pollen-burst in a species-specific manner (Amien et al., 2010).

Most antimicrobial peptides and defensins are developmentally regulated and are often expressed in a tissue-specific manner. This supports the possibility that these peptides serve dual roles, both as regulators of development and growth as well as antimicrobial action. As with the mode of action, there is still much research to be done to further investigate the importance of these peptides as having developmental roles.

2.5 AMPs and grapevine

Modern viticulture faces a number of challenges to maintain sustainable production of high quality grapes in a changing environment. Meeting these challenges requires an extensive understanding of the genetics and biology of the grapevine (*Vitis vinifera* L.), as well as interactions with its environment in the vineyard (Martìnez-Zapater et al., 2009). The potential of grapevine as a model organism for fruit trees has made it an important focal point for research on a genomics level, matching advances made with sequenced *Arabidopsis*, rice and poplar genomes (Troggio et al., 2008). The amount of genetic information available for grapevine has increased dramatically during the past decade (Troggio et al., 2008), including SNP-based markers (NCBI, http://www.ncbi.nlm.nih.gov), genetic maps constructed based on these markers (Troggio et al., 2007), the identification of quantitative trait loci (QTLs) for various traits (Xu et al., 2008), expressed sequence tags (ESTs) (da Silva et al., 2005; Moser et al., 2005; Peng et al., 2007) and associated proteomic and metabolic profiles (Sarry et al., 2004; Castro et al., 2005; Mattivi et al., 2006; Deluc et al., 2007; Deytieux et al., 2007; Young and Vivier, 2010).

The importance of grapevine and the scientific focus on this woody perennial have motivated the sequencing of the *V. vinifera* genome (Jaillon et al., 2007; Velasco et al., 2007). The availability of the genome sequence as well as rapidly evolving DNA techniques and technologies are providing tools that promise to revolutionize grapevine breeding techniques and support to grapevine research. Sequencing projects have provided researchers with information previously only available for model plant systems (Di Gaspero and Cattonaro, 2010).

This wealth of genomic information has made bioinformatics-coupled molecular approaches to gene discovery and the “mining” for potential genes of interest accessible to all researchers. Completed genomes sequences are available for a number of crop plants, including papaya (*Carica papaya*), maize (*Zea mays*), rice (*Oryza sativa*), apple (*Malus domestica*) and grapevine (*V. vinifera*) and these genomes are available on different databases that are publicly available (Table 5).

Table 2.5 Summary of publicly available databases providing searchable whole genome sequences (including crop plants).

Database	Website (http://)	Organisms	No. of available genomes
NCBI	http://www.ncbi.nlm.nih.gov/sites/genome	Prokaryotes, Eukaryotes	832
Plaza	http://bioinformatics.psb.ugent.be/plaza/	Plant	23
Genoscope	http://www.genoscope.cns.fr	Prokaryotes, Eukaryotes	8
IASMA	http://genomics.research.iasma.it/	Plant	2
Genome Database for Rosaceae (GDR)	http://www.rosaceae.org/	Plant	9

The availability of these databases facilitates the use of a number of bioinformatics tools for *in silico* screening for genes of interest, such as antimicrobial peptides. Plant peptides comprise almost 16% of all available AMP sequences in publicly available databases, including lipid transfer proteins, snakins, heveins, thionins and defensins. A simple search on any one of these databases will provide a number of both annotated and putative AMP-related sequences from plants (Clara Pestana-Calsa et al., 2010).

The publication of the complete *V. vinifera* genome, access to extensive EST databases and the availability of a variety bioinformatics tools and software has made it possible to screen the grapevine genome *in silico* for potentially new antimicrobial peptides.

Using sequences of known antimicrobial peptides, the *V. vinifera* genome can be screened for similar sequences. These sequences can further be investigated by searching for available EST data linked to the sequences. They can also be compared to other, similar peptides through amino acid alignment analysis. Putative antimicrobial peptides will show sequence and structure similarity to known antimicrobial peptides. This approach will undoubtedly become the first step in most antifungal peptide studies of plants with sequences genomes.

2.5.1 The isolation and characterization of the first grapevine defensin: *Vv-AMP1*

Through extensive analysis of sequence databases for novel plant defenses, a putative plant defensin encoding gene was identified in *V. vinifera* (De Beer, 2008; De Beer and Vivier, 2008). The gene was designated *Vitis vinifera* antimicrobial peptide 1 (*Vv-AMP1*). Analysis of the then recently released *V. vinifera* genome indicated the gene is present as a single copy on chromosome 1.

The size of the *Vv-AMP1* coding sequence was shown to be 234 bp, and the gene encodes a predicted 77 amino acid peptide. This peptide comprises of a 30 amino acid signal peptide and a 47 amino acid mature peptide. The genomic copy of the gene is 742 bp in size. Comparison with the cDNA sequences indicates the presence of a 508 bp intron interrupting the predicted signal peptide.

Alignment analysis indicated a high sequence homology on amino acid level to γ-thionins and comparative homology modeling of the deduced amino acid sequence revealed that the tertiary structure of Vv-AMP1 showed the typical defensin structure consisting of an α-helix and triple-stranded anti-parallel β-sheet, stabilized by disulfide linkages between eight cysteine residues.

The expression profile of *Vv-AMP1* revealed that the gene is expressed in a tissue-specific manner, with expression only detected in berries. Gene expression is also highly developmentally regulated, with expression induced upon berry ripening (specifically at the onset of berry ripening, or véraison, 11 weeks post-flowering). Expression levels remained high throughout the remaining berry ripening stages. Induction studies (via wounding, osmotic stress, infection stress and treatment with jasmonic-, salicylic- and abscisic acid) were unable to induce *Vv-AMP1* expression, either in leaf material or pre- véraison berries (De Beer and Vivier, 2008).

Sequence analysis predicted that the grapevine plant defensin would be localized to the apoplastic regions of plant tissues. Localization studies were carried out by fusing the Vv-AMP1 signal peptide to the green fluorescent protein GFP and constitutively overexpressed the fusion peptide in tobacco. Through fluorescent microscopy, it was confirmed that the signal peptide targeted the reporter gene to the apoplast (De Beer and Vivier, 2008), thereby confirming an apoplastic localization for Vv-AMP1.

Vv-AMP1, fused to a GST-tag, was recombinantly produced in *E. coli* using the Rosetta Gami pLysS expression system and glutathione affinity chromatography. Through mass spectrometry, the size of the recombinant peptide was determined to be 5,495 kDa. The antimicrobial activity of recombinantly produced Vv-AMP1 was tested against several pathogenic plant fungi using a dose-response growth inhibition assay (Broekaert et al., 1990). The peptide showed activity against a range of fungi, including *F. oxysporum* and *V. dahlia* with IC_{50} values of 6 µg/ml and 1.8 µg/ml respectively (De Beer and Vivier, 2008).

Microscopical analysis of treated fungal hyphae showed no signs of hyperbranching, though the ability of the hyphae to elongate was severely affected by the peptide. The peptide was thus categorized as a non-morphogenic peptide. A propidium iodide uptake assay showed that untreated fungi showed no fluorescence while treated samples showed high levels of fluorescence. This indicated the fungal membranes were compromised in the presence of Vv-AMP1 (De Beer and Vivier, 2008).

Despite the activity of the Vv-AMP1 peptide against a number of fungal pathogens, the overexpression of *Vv-AMP1* in tobacco did not reduce disease susceptibility towards *B. cinerea*. This could be attributed to either post-transcriptional modification of the Vv-AMP1 peptide or the instability of the peptide in the tobacco apoplast. With *Vv-AMP1* expression being so tightly regulated in its native host, *V. vinifera*, it has been suggested that the stability of the peptide is dependent on specific physiological conditions only present in the grapevine berries at the onset of berry ripening (De Beer, 2008; De Beer and Vivier, 2008).

Further investigation regarding the characteristics and function of this peptide, including evaluating the role of the peptide within its native host, as well as its effectiveness against typical grapevine fungal pathogens are still required and formed the base of this thesis (see Chapter 4).

2.6 Summary

Our knowledge and understanding of host defense systems has rapidly expanded over the past decade. The importance of antimicrobial peptides in host defense has become clear and the potential of these peptides have become the focus of biotechnological research, for both clinical and agricultural purposes.

Antimicrobial peptides have been isolated from diverse groups of organisms, including invertebrates, vertebrates, insects and plants. These peptides are classified into different categories based on their size, conformational structure and amino acid composition. Of all the antimicrobial peptides isolated in plants, the majority are typically plant defensins.

The exact mechanism of antifungal action of plant defensins is not entirely understood, but it is generally accepted that the peptides act on the fungus at the level of the plasma membrane. It has also been shown that some defensins such as NaD1 require interaction with the fungal cell wall in order to permeabilize the plasma membrane. This suggests that NaD1 permeabilizes the cell membrane in a novel way, utilizing a receptor which is possibly located in the proteinaceous layer of the cell wall (van der Weerden et al., 2010). Other studies suggest that many defensins exhibit a dual role in general plant development.

With the rapid increase in available genome sequences for plants and other organisms, as well as extensive databases for ESTs and antimicrobial peptides, the discovery of novel antimicrobial peptides has not only increased exponentially, but allowed for more targeted approaches.

One such discovery was the first antimicrobial peptide in grapevine, Vv-AMP1, (with the possibility of several more AMPs of different classes found in the genome). The discovery, isolation and characterization of Vv-AMP1 have enabled further investigation of the biological role of the peptide within its native host.

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Chapter 3

Research Results

Heterologous production of Vv-AMP1, a defensin from grapevine, in *Escherichia coli*

RESEARCH RESULTS

Heterologous production of Vv-AMP1, a defensin from grapevine, in *Escherichia coli*

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3.1 Abstract

Antimicrobial peptides occur in many living organisms and isolation, characterization and functional analyses of a wide spectrum of these peptides have confirmed their importance in defense systems of their respective hosts. The recombinant production of antimicrobial peptides in heterologous expression systems presents a multitude of obstacles and often requires fusing the mature peptide to a tag-protein to prevent the peptide from showing activity during production. Here we report on the recombinant production of Vv-AMP1, a defensin peptide previously isolated from grapevine. We show that with specific optimization we could improve the yield of mature Vv-AMP1 significantly by systematically evaluating and optimizing several steps of the induction and purification procedure. The optimized purification method yielded up to 3 mg of pure Vv-AMP1 peptide from 1.6 L of an overnight culture. The identity and integrity of the peptide was confirmed through SDS-PAGE and Western blot analysis. However, the recombinantly produced Vv-AMP1 was inactive against two test organisms known to be very sensitive to Vv-AMP1. Previous *in vitro* studies confirmed activity of Vv-AMP1 against *Botrytis cinerea* at very low concentrations, but the bacterially produced Vv-AMP1 did not show any activity against this organism in liquid plate assays, even at excessively high concentrations. The results suggest that an important aspect for peptide activity such as folding, or perhaps specific co-factors are not supported in the non-host prokaryotic production system used in this study.

3.2 Introduction

Antimicrobial peptides are biologically active molecules that form part of host immunity. They have been identified and isolated from a variety of sources, including mammals, plants, insects, amphibians, fish and prokaryotes (Andreu and Rivas, 1998; Hancock and Lehrer, 1998; Izadpanah and Gallo, 2005). These peptides often have a broad range of activity against bacteria and fungi, while having minimal toxic effect on the host (Lehrer et al., 1993; Rao, 1995; Reddy et al., 2004). Their potent anti-pathogenic characteristics make them promising targets for research in the pharmaceutical, medical and crop biotechnology sectors.

Current methods of peptide production are generally limited and often production yields are very low whereas large quantities of peptide are required to evaluate the characteristics of the peptides. Small peptides can be efficiently produced through chemical synthesis, but this requires specific expertise and equipment (Bruckdorfer et al., 2004). Small, cysteine-rich peptides such as defensins often occur in multigene families. Their conserved structural features make it difficult to purify a specific peptide from a pool of very similar peptides found in the source organs or species (Ingham and Moore, 2007). If the sequence of the peptide is known, heterologous production can facilitate the production of a specific peptide.

Recombinant methods allow the production of peptides in expression systems such as yeast or bacteria (Zorko et al., 2009). With the field of peptidomics expanding rapidly, the demand for efficient, relatively simple methods of recombinant protein production has increased greatly in recent years (Terpe, 2003).

A well developed method of recombinant protein production typically involves the use of affinity-tags (Makrides, 1996). The basic features of affinity-tag systems include the fusion of an affinity-tag to the target peptide, simple adsorption purification, easy and specific removal of the affinity-tag and straightforward recovery of the purified peptide. A variety of affinity-tag systems are commercially available, including polyarginine-tags (Arg-tag), polyhistidine tags (His-tag), Strep-tag, calmodulin-binding peptide (CBP), maltose-binding protein (MBP) and glutathione S-transferase (GST) tags (Terpe, 2003).

Affinity-tag purification, however, often affects important characteristics or functions of the protein or peptide it is fused to. Removal of the tag is generally necessary and achieved through cleaving with a site-specific protease such as thrombin, enterokinase or Factor X_a (Ingham and Moore, 2007). Other systems rely on intein-mediated purification, which allows cleavage of the tag without protease activity, but rather through a self-splicing intein (Morassutti et al., 2002; Sharma et al., 2006).

The expression of antimicrobial peptides in microbial systems poses some difficulties, the most apparent being the potential cytotoxicity of the produced active peptide to the host cells (Jin et al., 2006; Xu et al., 2007). Recombinant production of these peptides is made possible by using affinity-tag systems. The fusion of the antimicrobial peptide to a partner- or tag-protein renders the peptide inactive, allowing production with no toxic effects to the host cells (Wei et al., 2005). Once the peptide is released from the fusion protein through chemical or enzymatic cleavage, it should regain its activity (Ingham and Moore, 2007).

Despite the development and improvement of these recombinant peptide production systems, producing and purifying of antimicrobial peptides still present a number of challenges. Extensive knowledge and understanding of the target protein or peptide is required to individually optimize each step in the production process. Working with small peptides is often difficult, demanding stable, controlled and highly repeatable conditions. Simple, technical aspects related to the type of material used, the preparation of the consumables and even the environmental conditions during the experiment could have an influence on peptide production, yield and even stability.

Previously, the first grapevine defensins, Vv-AMP1, was isolated, purified and characterized. *In vitro* assays indicated it was a non-morphogenic peptide which was able to disrupt the membranes of fungal pathogens (De Beer and Vivier, 2008).

In this previous study, Vv-AMP1 was overexpressed and purified in an active form using the pGEX bacterial overexpression system, but relatively low yields were obtained (De Beer and Vivier, 2008). The aim here was to systematically optimize induction and purification to increase yields of active Vv-AMP1 peptide to use in activity assays against a range of grapevine pathogens. A consistent method of production of Vv-AMP1 was undertaken through a number of optimization steps (outlined in Table 3.1), but despite having increased yields, the peptide produced was not active against organisms known to be sensitive against Vv-AMP1. The results suggest that the optimized production system negatively impacted on a crucial aspect required for activity of this peptide.

3.3 Materials and Methods

3.3.1 Microbial and fungal pathogen strains

For recombinant protein production, *Escherichia coli* strain BL21 (Rosetta-gami pLys S) DE3 (Novagen (Madison, WI, USA) was used. Cultures were maintained as freeze cultures containing 50% (v/v) glycerol and stored at -80°C. *Botrytis cinerea* was obtained from the Institute for Wine Biotechnology (IWBT). Cultures were maintained on sterile apricot halves in corning tissue culture plates at 25°C until sporulation. Spores were harvested in sterile dH_2O, 24 h prior to use and hydrated overnight at 4°C.

3.3.2 Transformation of *E. coli* with GST expression vector

The pGEX-2T system (Amersham Biosciences, NJ, USA) was used for the recombinant production of Vv-AMP1 in *E. coli*. The system would produce mature Vv-AMP1 peptide fused to the GST-tag. The pGEX-Vv1 expression vector was constructed as described in De Beer and Vivier (2008). The integrity of the construct was verified through sequencing (Central Analytical Facility, University of Stellenbosch) and the construct was found to contain the correct sequence of the *Vv-AMP1* gene.

The confirmed pGEX-Vv1 vector was transformed into the BL21 strain of *E. coli* using a heat shock transformation method. 50 ng of pGEX-Vv1 DNA was added to a 100 µl aliquot of BL21 cells. The mixture was incubated on ice for 1 h. Thereafter it

was heat shocked for 45 s at 37ºC and incubated on ice for 2 min. 1 ml LB liquid medium (Merck, NJ, USA) without antibiotics was added and mixture was incubated at 37ºC for 60 minutes.

Cells were plated out on selective media of LB agar containing 50 $\mu g.ml^{-1}$ ampicillin, 34 $\mu g.ml^{-1}$ chloramphenicol (Roche Diagnostics GmbH, Mannheim, Germany), 12.5 $\mu g.ml^{-1}$ tetracyclin and 15 $\mu g.ml^{-1}$ kanamycin sulphate (Sigma, St. Louis, USA) and. Plates were incubated overnight at 37ºC. Positive colonies were picked, inoculated in selective liquid media and incubated overnight at 37ºC. Freeze cultures were made by adding 1 ml of overnight culture to 1 ml 80% (v/v) glycerol. Freeze cultures were stored away at -80ºC.

3.3.3 Standard method of recombinant production and purification of Vv-AMP1

The standard method of recombinant production and purification of Vv-AMP1 was followed as described by De Beer and Vivier (2008). This method was used for comparison with the optimized induction and production method (see section 3.3.4).

3.3.4 Optimized induction, production and purification of Vv-AMP1

Growth conditions and other basic steps were followed as described by De Beer and Vivier (2008). The specific optimizations refined for Vv-AMP1 production are summarized in Table 1.

5 ml LB growth medium containing antibiotics (50 $\mu g.ml^{-1}$ ampicillin, 12.5 $\mu g.ml^{-1}$ tetracyclin, 15 $\mu g.ml^{-1}$ kanamycin sulphate and 34 $\mu g.ml^{-1}$ chloramphenicol) was inoculated from a freeze culture of *E. coli* BL21 containing the pGEX-Vv1 construct and grown overnight at 37°C. One ml preculture was inoculated into four 1 L Erlenmeyer flasks, each containing 400 ml of LB growth medium with antibiotics. The cultures were grown at 37°C with continuous shaking until an OD_{600} of 0.7 was reached. Expression of the GST-VvAMP1 fusion peptide was induced with 0.4 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Roche Diagnostics GmbH, Mannheim, Germany) for 5 hours at room temperature. Bacterial pellets were harvested by centrifugation (10 min, 5000 x g 4°C); pellets were resuspended in 10 ml GST binding buffer (300 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and frozen at -80°C. Cells were disrupted through 6-8 repetitive freeze-thaw cycles

from liquid nitrogen to a 37°C waterbath. 5 mM $MgCl_2$ was added to and lysate was treated with 10 units of DNase I (Roche Diagnostics GmbH, Mannheim, Germany) for 15 minutes at room temperature to reduce viscosity. Triton x-100 was added to a final concentration of 1% (w/v). Lysate was centrifuged at 10 000 rpm for 15 minutes, clear lysate was removed and passed through a 0.22 µM filter.

Table 3.1. Systematic optimization steps undertaken to improve production and purification of Vv-AMP1 from the pGEX bacterial overexpression system.

Steps in the overproduction and purification procedure targeted for optimizations	**Aspects evaluated**	**Parameter optimized**
Induction of peptide production by the bacterial culture	Optimal conditions and length of induction with IPTG	Induction temperature (37°C versus room temperature) and induction time (overnight versus 5 hours)
Purification	Preparation and binding of lysate	Composition of binding buffer (135 mM versus 300 mM) Shorter binding time (overnight versus 1 hour)
	Washing of column	Optimal wash volume (5 column volumes versus 10 column volumes)
	Elution	Concentration of reduced glutathione (10 mM versus 50 mM)
	Thrombin cleavage	Efficiency of different enzymes (Novagen thrombin protease versus Amersham thrombin protease)

Prepared lysate was added to a 2 ml bed volume of Glutathione agarose 4B (Sigma, St Louis, USA). Recombinant peptide was allowed to bind to the agarose for 4 hours on a rotor mixer. Unbound proteins were removed by washing the column twice times with 10 ml GST wash buffer (50 mM Tris-HCl pH 7.5, 0.25 mM NaCl, 2 mM EDTA) and once with 10 ml GST wash buffer containing 1% Triton x-100 to remove unspecific proteins. Bound recombinant GST-VvAMP1 peptide was eluted with 15 ml elution buffer (50 mM Tris-HCl, pH 8.0, 50 mM reduced glutathione). The N-terminal GST tag was removed with 20 units of thrombin protease enzyme (Amersham Biosciences, NJ, USA) digested overnight at room temperature.

3.3.5 Size determination, identification and immunoblotting of heterologously produced Vv-AMP1

Confirmation of Vv-AMP-GST fusion proteins and evaluation of cleavage of Vv-AMP1 from the GST-tag were achieved through separation on a 15% [w/v] tricine-sodium dodecyl sulfate-polyacrylamide gel (Schägger & von Jagow, 1987). After separation, the gels were microwaved in a staining solution (Coomassie R250 dissolved in 50% [v/v] ethanol and 10% [v/v] acetic acid) until the solution boiled. Thereafter, gels were placed on a shaker for 30-60 minutes. After sufficient staining, the gels were placed in a destaining solution (12.5% [v/v] isopropanol and 12% [v/v] acetic acid) until the background was removed and the bands were clearly visible.

Western blot analyses were conducted on samples of putatively purified Vv-AMP1. A sample of confirmed purified Vv-AMP1 peptide (obtained from Dr. A. De Beer) was always present as a positive control. The samples and a low molecular weight marker (Sigma, St Louis, USA) were separated on a 15% (w/v) Tricine-SDS-PAGE (Schägger & von Jagow, 1987). The membrane was electroblotted to PVDF membranes (BioRad, Hercules, CA, USA) for 30-60 minutes (400 V, 75 mA). Membranes were carefully removed from the electroblotting apparatus and placed in blocking reagent (5% skim milk powder dissolved in Tris-buffered saline and 0.1% [w/v] Tween 20) for 1 hour, at room temperature. Membranes were rinsed with 2 changes of wash buffer (0.1 % [w/v] Tween 20 in Tris-buffered saline) before being incubated for 1 hour in a 1:5000 dilution of primary antibody prepared in blocking buffer. Primary antibodies (obtained from Dr. A. De Beer) were polyclonal antibodies, produced in mice against the GST-VvAMP1 fusion protein. Membranes were rinsed for 15 minutes in wash buffer, followed by three 5-minute washes. Thereafter the

membranes were incubated for 1 hour with anti-mouse IgG secondary antibody (Sigma, St Louis, USA). Membrane wash steps were repeated as described. The wash buffer was discarded and detection of Vv-AMP1 was completed using the ECL chemiluminescent system according to Amersham Biosciences, NJ, USA.

3.3.6 Antimicrobial activity of recombinant Vv-AMP1

The quantitative antifungal activity of the antifungal peptides was measured using a microspectrophotometric antifungal assay (Broekaert et al., 1990). The assay was performed in a 96 well microtiter plate (Nunc). Each individual reaction contained 100 µl half strength potato dextrose broth (Merck, NJ, USA) with 2000 fungal spores and a range of peptide concentrations. The control wells contained no peptide. The plates were incubated in the dark at 25°C for 2 days. Microspectrophotometric readings were taken every 24 hours at 595 nm (PowerwaveX microplate reader, Bio-Tek instruments inc.).

Measurements (A_{595}) were taken at 0 h, 24 h and 48 h. Values were corrected by subtracting the 0 h value from the 24 h and 48 h measurements. Activity is expressed in terms of the percentage growth inhibition and can be calculated with the formula:

$$\%\,Inhibition = 100 - \left(\frac{Corrected\ (A_{595})_{CONTROL} - Corrected\ (A_{595})_{SAMPLE}}{Corrected\ (A_{595})_{CONTROL}} \times 100 \right)$$

3.4 Results

3.4.1 Induction of peptide production

Induction of the bacterial culture to initiate production of the recombinant GST-VvAMP1 peptide was achieved through addition of IPTG. Preliminary experiments indicated that the level of production of the fusion peptide was directly affected by both temperature and length of induction (results not shown). Yields were improved by a lower induction temperature and shorter induction times (results not shown). Optimal induction conditions proved to be at room temperature for 5 hours on an orbital shaker (Figure 3.1). Figure 3.1(B) shows an increased production of the fusion peptide (~31 kDa) under optimized conditions when compared to the standard method (overnight at 37ºC).

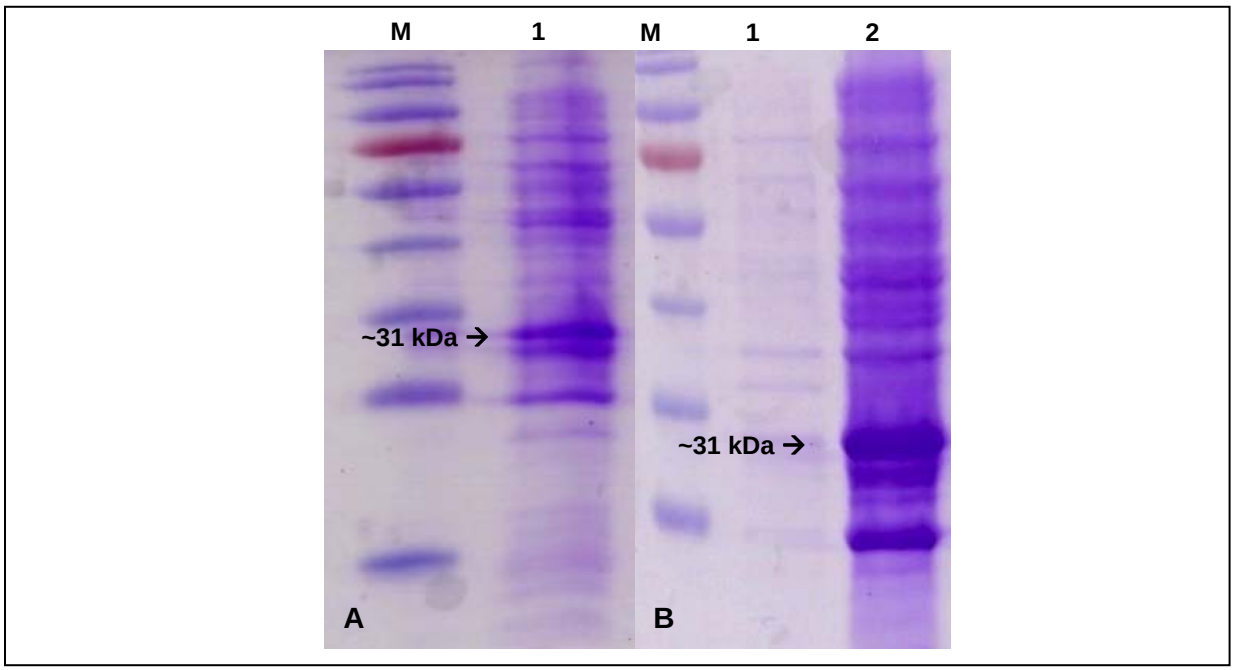

Figure 3.1 SDS-PAGE analysis comparing recombinant protein production between standard (A) and optimized (B) methods. In panel A: Lane M – 5 µl PageRuler ™ prestained protein ladder (Fermentas); Lane 1 – 5 µl of sample induced overnight at 37°C ; In panel B: Lane M – 5 µl PageRuler ™ prestained protein ladder (Fermentas); Lane 1 – 5 µl of sample before induction; Lane 2 - 5 µl of sample induced at room temperature for 5 hours.

3.4.2. Purification

Subsequent purification steps to isolate the fusion peptide and to separate Vv-AMP1 from the GST-tag and the protease required optimization at a number of stages (see

Table 1). Binding of the lysate to the affinity column was improved by increasing the concentration of salt in the buffer from 135 mM to 300 mM (results not shown). This reduced the amount of non-specific proteins binding to the Sepharose 4B column. Overnight binding of the lysate to the column proved excessive as 1 hour of binding proved sufficient to bind all the GST-VvAMP1 peptide to the column. In Figure 3.2(A), lane 2 represents a sample of the flow-through after overnight binding of the lysate to the Sepharose 4B column. Figure 3.2(B), lane 3 represents a sample of flowthrough after binding of the lysate to the column for only 1 hour. The SDS-PAGE analysis of the sample indicates that all the produced fusion peptide is sufficiently bound to the column within 1 hour.

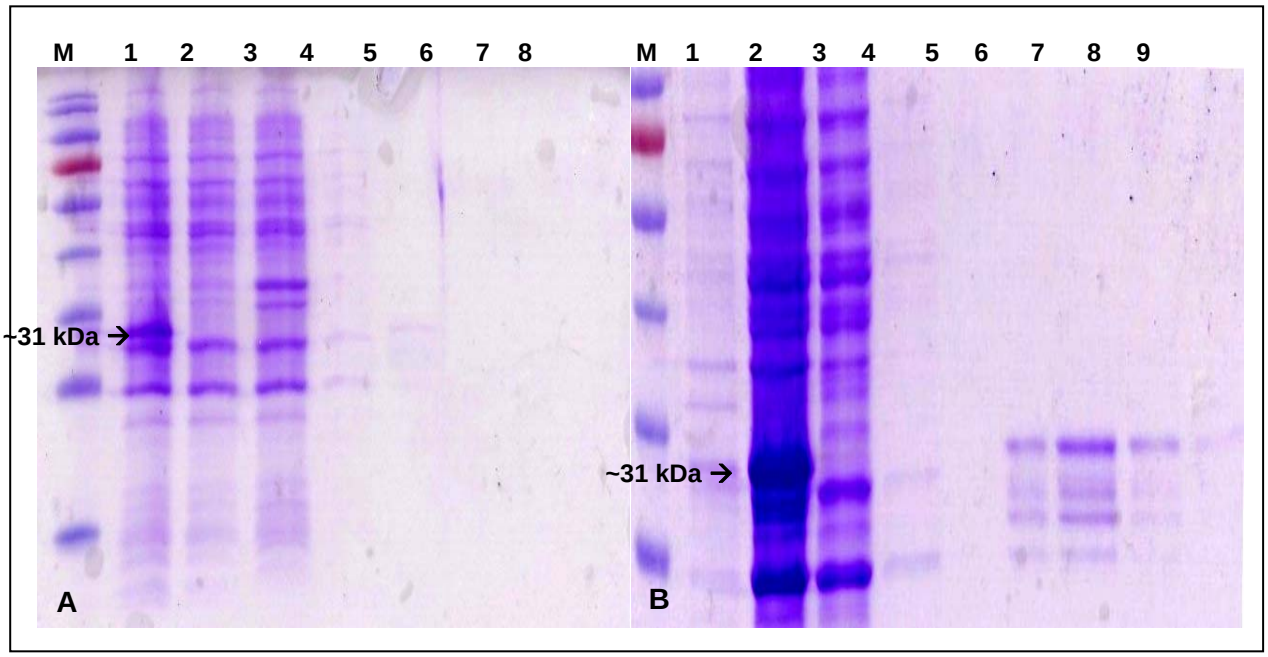

Figure 3.2 SDS-PAGE analysis comparing different binding conditions, wash volumes and elution conditions. In panel A: Lane M – prestained protein ladder (Fermentas); Lane 1 – 5 µl sample of lysate (post-induction) before binding to Sepharose 4B; Lane 2 – 5 µl sample of flowthrough after overnight binding to Sepharose 4B; Lane 3 – 10 µl sample of flowthrough after overnight binding to Sepharose 4B; Lane 4 and 5 - samples of column wash; Lanes 6-8 – fractions of elution with reduced glutathione. In panel B: Lane M – prestained protein ladder (Fermentas); Lane 1 – 5 µl sample of lysate before induction with IPTG; Lane 2 – 5 µl sample of lysate (post-induction) before binding to Sepharose 4B; Lane 3 – ample of flowthrough after binding to Sepharose 4B column (1 h binding time); Lane 4 and 5 – 5 µl of consecutive samples of column wash flow-through; Lanes 6-9 – fractions of elution with reduced glutathione.

Washing the column to remove unbound proteins and other impurities was improved by increasing the wash volume from 5 column volumes to 10 column volumes. In Figure 3.2(A), lane 4 contains a sample of the column wash flowthrough using 5 column volumes of wash buffer. Lane 5 contains a sample of flowthrough after an additional column volume of wash buffer was added to the column. This sample still contained traces of non-specific proteins. In Figure 3.2(B), lane 4 represents the sample of column wash flowthrough using 10 column volumes of wash buffer. Lane 5 represents a sample of flowthrough after an additional column volume of wash buffer was added to the column. This lane contains no detectable non-specific proteins.

Elution of the GST-VvAMP1 fusion peptide from the Sepharose 4B affinity column was achieved by adding 10 mM reduced glutathione to the elution buffer; however initial glutathione elutions were unsuccessful, yielding little or no fusion peptide in the elution fractions. In Figure 3.2(A), the elution fractions (lanes 6-8) contain no eluted GST-VvAMP1 peptide when eluted with 10 mM of reduced glutathione. Increasing the reduced glutathione to 50 mM proved successful (Figure 3.2(B), lanes 6-9).

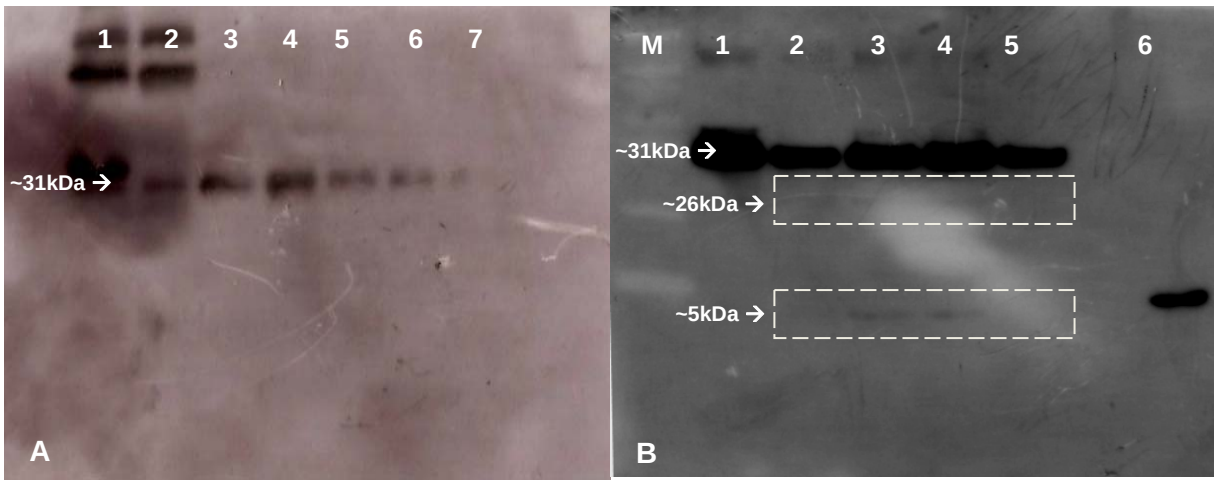

Figure 3.3 Western blot analyses indicating the presence of GST-VvAMP1 after production and elution, comparing the cleaving efficiency of thrombin protease enzyme from Novagen and Amersham. In panel A: Lane 1 - sample of flowthrough after induction with IPTG, before binding to Sepharose 4B; Lane 2 - sample of flowthrough after binding to Sepharose 4B; Lanes 3-7 – elution fractions containing GST-VvAMP1 fusion peptide (~31 kDa) and no cleaved Vv-AMP1 after overnight digestion with Novagen thrombin protease. In panel B: Lane M – prestained protein ladder (Fermentas); Lane 1 – sample containing undigested GST-VvAMP1; Lanes 2-5 – elution fractions containing GST-VvAMP1 fusion peptide, GST and cleaved Vv-AMP1 after digestion with Amersham thrombin protease; Lane 6 – purified Vv-AMP1 as positive control.

The presence of the GST-VvAMP1 fusion peptides (~31 kDa) in the elution fractions were confirmed through Western blot analyses. The fusion peptide was present as a clear band of approximately 31 kDa in size (Figures 3.3 A and B). In Figure 3.3(A), lanes 1 and 2 represent samples of the lysate before and after binding to the column, respectively. A large ~31 kDa band is clearly visible in the sample in lane 1, while the band is much less pronounced in lane 2, indicating the removal of a large amount of the GST-VvAMP1 fusion peptide from the sample through binding to the column. Binding of the fusion peptide is limited by the binding capacity of the Sepharose 4B column. Lanes 3 to 7 indicate the presence of the fusion peptide in the elution fractions collected.

Thrombin cleavage was evaluated using enzymes acquired from both Novagen and Amersham. Cleavage using both enzymes was attempted using 20 units of enzyme in 1 x PBS buffer for 16 - 22 hours at room temperature. The thrombin from Amersham proved far more efficient in cleaving the Vv-AMP1 from the GST-tag (Figure 3.3 B, lanes 2-5), though still only partially.

Separation of the GST-tag from Vv-AMP1 through size exclusion chromatography on a Sephadex G-25 column clearly indicated the Vv-AMP1 peptide co-eluting with a salt fraction (Figure 3.4) when samples are digested in 1 x PBS. Desalting can be achieved using C8 columns, but this caused a notable decrease in peptide yield (results not shown). Alternatively, thrombin digestion in sodium acetate buffer (a volatile salt) allowed the addition of 100% acetonitrile to the sample, which facilitates removal of the GST-tag through denaturation. The sodium acetate can be easily removed through several cycles of freeze-drying. Concentration determinations revealed that up to 3 mg/ml of pure Vv-AMP1 peptide could be produced from 1.6 L of starting culture using this method.

Purified putative Vv-AMP1 was tested against *B. cinerea* at a range of concentrations, from 10 µg/ml to 25 µg/ml. No significant antimicrobial activity was observed (Figure 3.5). Previously, 50% inhibition of *B. cinerea* growth was achieved in the presence of 13 µg/ml of purified Vv-AMP1 (De Beer and Vivier, 2008).

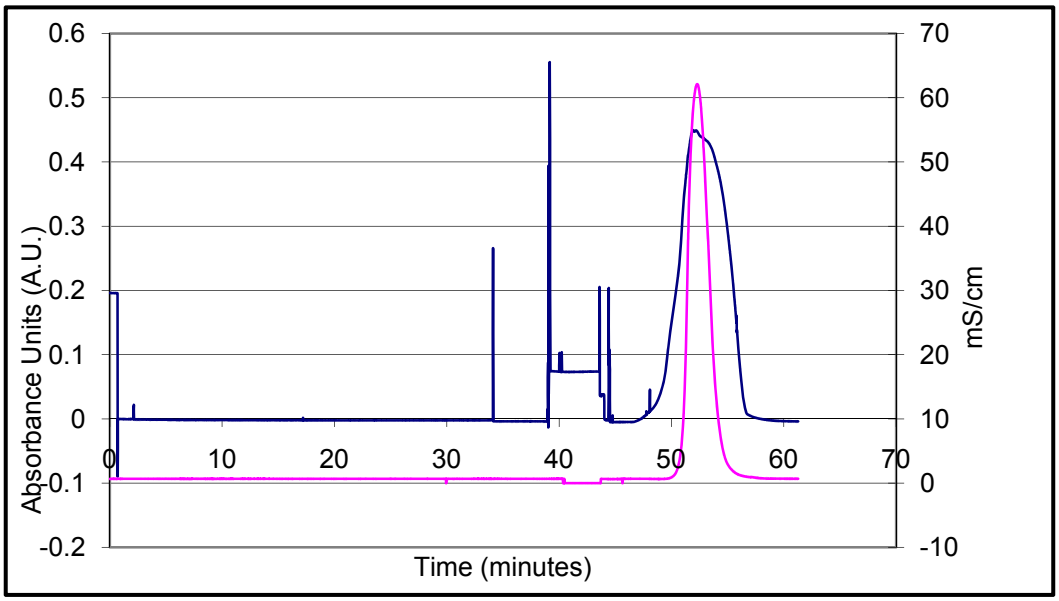

Figure 3.4 Chromatogram indicating co-elution of Vv-AMP1 fraction with salt during seperation through size exclusion chromatography on Sephadex G-25 (BioRad Biologic LP chromatography system). The blue line represents the UV measurement (measured in Absorbance Units) indicating the elution of the peptide off the Sephadex G-25 column. The pink line represents measured conductivity (mS/cm) indicating the presence of salt in the fraction. The chromatogram shows the overlapping of the two peaks, indicating the co-elution of the peptide with salt.

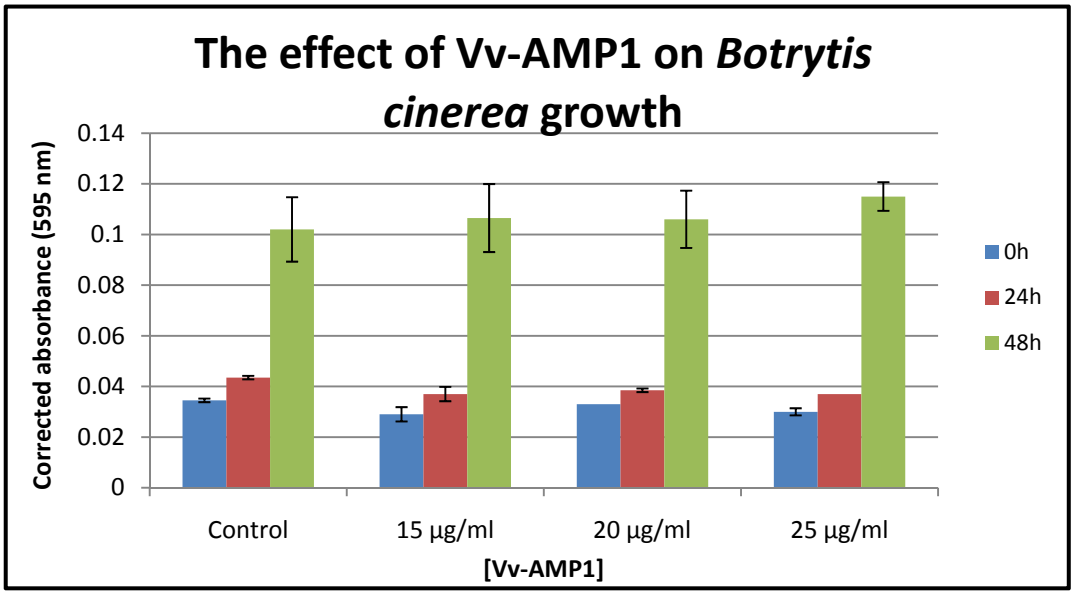

Figure 3.5 Antifungal assay evaluating the ability of purified Vv-AMP1 to inhibit the growth of the grapevine pathogen *Botrytis cinerea* at different concentrations over a period of 24 hours. Results show no significant inhibition of fungal growth, even in the presence of extremely high concentrations of the putative Vv-AMP1 peptide.

3.5 Discussion

The production of biologically active antimicrobial peptides are always challenging when using microbial production systems (Ingham and Moore, 2007). *E. coli* is often the preferred host organism for a recombinant protein production system as it offers several advantages: *E. coli* is genetically very well characterized and understood, with many tools available to facilitate genetic engineering through gene cloning and expression. Despite the extensive knowledge of this Gram-negative bacterium, there can be no guarantee that every gene will be successfully expressed within an *E. coli* expression system (Makrides, 1996; Schumann and Ferreira, 2004). Failure of recombinantly produced proteins to be effectively produced or to be biologically active can be attributed to factors such as incorrect folding, proteolytic degradation or associations with each other forming inclusion bodies (Baneyx and Mujacic, 2004).

The efficiency of such recombinant protein production systems are evaluated by considering factors such as speed of production, yield, purity, stability of the resultant protein and experimental reproducibility. Though commercially available systems are designed to be simple to use and easy to modify, the production process must be empirically evaluated and optimized accordingly, depending on the nature and qualities of the specific peptide produced (Schumann and Ferreira, 2004).

Smaller peptides may prove particularly problematic, specifically with regards to retaining their activity as shorter peptides are often rapidly degraded by proteases in *E. coli* (Douglas Fairlie et al., 2002). Production systems utilizing tag-proteins (such as the GST-tag) are especially effective for producing small biologically active antimicrobial peptides by rendering them inactive when fused to the much larger tag protein.

Vv-AMP1, the first defensin isolated from grapevine, is a small, cysteine-rich peptide. This 77-amino acid shows strong similarity to plant defensins and is expressed in the berry only at the onset of berry ripening. The tertiary structure of Vv-AMP1 reveals a typical defensin structure, consisting of an α-helix with a triple-stranded anti-parallel β-sheet. These are stabilized by disulfide linkages between eight cysteine residues. The peptide is non-morphogenic and treated fungi show no signs of hyperbranching but it has a compromising effect on fungal membranes. Vv-AMP1 is stable even at high temperatures, retaining up to 62% activity after treatment for 30 minutes at 100ºC. The peptide inhibited the growth of different plant pathogenic fungi at very low concentrations. (De Beer and Vivier, 2008).

Production of the Vv-AMP1 peptide had previously been attempted using the His-tag fusion system. This method proved unsuccessful, as it seemed the His-tag was insufficient to inhibit the activity of the peptide and upon induction, the produced peptide negatively impacted the *E. coli* cells producing it, leading to extremely low yields (personal communication, Dr. Abrè de Beer, IWBT, Stellenbosch University). Subsequent production of Vv-AMP1 was achieved successfully, resulting in biologically active peptide, but at very low yields (De Beer and Vivier, 2008). Improving production of Vv-AMP1 with the GST-tag fusion system to increase both yield and purity of the peptide required meticulous refinement of each step in the production process.

The system proved sensitive to slight changes in growth and induction conditions, and optimization of the first step was necessary to ensure adequate production of the fusion protein. Also, while induction at high cell densities and for extended periods might increase the yields of the fusion protein, it risks the formation of inclusion bodies (Saluta and Bell, 1998). With this in mind, growth and induction parameters were varied by lowering the induction temperature, shortening the induction time and increasing the aeration of the culture during induction. Induction proved optimal for 5 hours at room temperature, leading to a marked improvement in production of the fusion peptide. Both shorter and longer induction times led to a decrease in peptide production, as did induction at higher temperatures (e.g. 37°C).

The binding of GST to the glutathione Sepharose 4B is a highly specific interaction. This reduces the occurrence of non-specific interactions by other proteins and peptides with the sepharose. When non-specific binding was observed, increasing the salt concentration of the binding buffer to 300 mM served to reduce it. Increasing the volume of wash buffer used also aided in removing unwanted proteins and substances interacting with the column.

The inefficient elution of the GST–VvAMP1 fusion peptide was addressed by increasing the concentration of reduced glutathione in the elution buffer from 10 mM to 50 mM. Enzymatic removal of the GST tag through thrombin protease digestion was improved by using a more efficient thrombin, acquired from Amersham Biosciences. Both manufacturers' thrombin proteases should be suitable to cleave the Vv-AMP1 from the GST tag, but the Amersham thrombin proved much more efficient when compared to that of Novagen. Still, cleavage was sub-optimal, with large amounts of uncleaved GST-VvAMP1 still present in samples after overnight digestion. This inefficient cleavage may be explained by sub-optimal cleavage conditions and requires further investigation to ensure optimal cleavage and complete removal of Vv-AMP1 from the GST tag.

The production and purification of the GST-VvAMP1 fusion peptide was markedly improved through the optimizations investigated in this study, leading to a noticeable increase in yield of purified peptide when compared to the standard method of production. De Beer and Vivier (2008) reported up to 5 mg/L of purified recombinant peptide, while this study yielded nearly double that. Despite this improvement in initial yield, the cleavage of the Vv-AMP1 peptide remained problematic. When the purified peptide was tested against fungal pathogens known to have their growth inhibited by Vv-AMP1, the peptide showed no inhibitory activity.

The purified Vv-AMP1 also exhibited uncharacteristic instability, showing signs of degradation within a week of purification, even when stored under optimal conditions. This is in contrast to the results obtained regarding the intrinsic stability of the purified peptide, as reported by De Beer and Vivier (2008).

Both the inactivity and instability of the resultant peptide could be a result of incorrect folding of the peptide after cleavage. This would render the peptide unable to inhibit antimicrobial growth as normal and also affect the stability of the peptide, if the incorrect folding affects or changes the peptide structure. Incorrect folding of the peptide may be ascribed to the absence of necessary co-factors or molecular chaperones that are not present in the prokaryotic intracellular environment. Though the *E. coli* strains used in recombinant expression systems are engineered to reduce the occurrence of codon bias, the organism's ability to produce the desired peptide might still be inhibited, especially if the gene expressed is rich in codons rarely used by *E. coli*, codons such as AGA and AGG, coding for arginine (Makrides, 1996).

For the optimizations described in this study regarding improved production to be meaningful, it is imperative that issues around cleavage and removal of the tag, peptide inactivity and peptide instability be further investigated and suitably addressed. Cleavage efficiency must be explored, specifically looking at cleavage conditions other than optimal temperature and length of cleavage. Other conditions such as amount of thrombin protease used per mg of fusion protein or the composition of the cleavage buffer may have an effect on the efficiency of the cleavage (Jenny et al., 2003).

After cleavage of the Vv-AMP1 peptide is optimal and yields of purified, cleaved peptide are increased, the activity and stability of the resultant peptide can be re-evaluated. Alternatively, if these issues remain unsolved, it might be prudent to investigate the use of a different expression system to circumvent the difficulties currently presented by using the GST-fusion system.

Recombinant expression systems utilizing eukaryotic host organisms (such as the *Pichia pastoris* expression system) may aid in overcoming problems of codon bias and the lack of molecular chaperones to ensure the correct amino acid sequence and subsequent correct folding of the eukaryotic gene being expressed. Other expression systems make use of non-enzymatic methods to cleave the tag from the target protein, some through the addition of dithiothreitol (DTT) to induce self-cleavage of intein splicing elements present in the fusion protein; others induce cleavage through a change in temperature or pH. These systems eliminate lengthy enzymatic cleavage steps from the protocol, which reduces the risks of enzymatic degradation and destabilizing of the protein.

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Chapter 4

Research Results

Vv-AMP1, a defensin from grapevine, shows strong antifungal activity *in vitro* and overexpression in grapevine slightly improves *in planta* resistance against *Botrytis cinerea*

This chapter will be incorporated into a manuscript that will be submitted to **Transgenic Research** after some additional infection studies have been completed.

RESEARCH RESULTS

Vv-AMP1, a defensin from grapevine, shows strong antifungal activity *in vitro* and overexpression in grapevine slightly improves *in planta* resistance against *Botrytis cinerea*

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4.1 Abstract

The importance of cysteine-rich, small antimicrobial peptides in the pre-existing defense system of plants is becoming increasingly apparent. Plant defensins are ubiquitous defense peptides in the plant kingdom and are an integral part of the innate immune system. *Vitis vinifera* antimicrobial peptide 1 (*Vv-AMP1)* is the first plant defensins isolated from grapevine. This 77 amino acid peptide is only expressed in berries at the onset and duration of berry ripening. The peptide showed strong activity against a range of fungal pathogens, including *Botrytis cinerea*, *Fusarium oxysporum* and *Verticillium dahliae*. Here we further investigated the functional characteristics of the peptide, both *in vitro* and *in planta*. In *in vitro* antifungal assays, Vv-AMP1 showed strong activity against the following grapevine pathogens: *Phomopsis viticola*, *Diplodia seriata*, *Phaemoniella chlamydospora*, and *Cylindrocarpon liriodendri*. Microscopic analysis of peptide-treated fungal cultures confirmed a strong membrane disruption activity by the peptide. The peptide was further evaluated for its *in planta* role by overexpression in its native host. Putative transgenic lines of *V. vinifera* (cv. Sultana), transformed with an expression cassette containing *Vv-AMP1* and a constitutive promoter, were obtained and the presence and expression of the transgene could be confirmed in nine independently transformed lines. Some of the lines exhibited abnormal growth in the *in vitro* as well as the hardened off state. Leaf shape and growth point development was affected in some lines. In a first analysis of the *in planta* effect of *Vv-AMP1* in its native host, the

transgenic lines were evaluated by challenging the population with *B. cinerea* using a detached leaf infection assay. Symptom development proceeded over a six day period and statistical evaluation of the data showed that the transgenic lines behaved as a population and could not be distinguished from each other in terms of lesion sizes, but all lines could be separated from the untransformed control.

4.2 Introduction

The management and prevention of disease in crops has become a global industry that is continually searching for new and improved methods of controlling the incidence and spread of disease. Grapevine has been one of the world's most cultivated and economically important food crops for centuries. The importance of finding ways to improve the resistance of the crop to disease is significant (Troggio et al., 2008). The wine and table grape industry is faced with a considerable number of pathogens that specifically attack grapevine. For many of these diseases an effective method of prevention and/or control has yet to be developed.

It is known that plants have complex defense systems to recognize pathogens and to mount protective measure against invading pathogens. Understanding these pathways and the roles of the proteins and metabolites involved is paramount to develop efficient strategies for pathogen control. Functional analysis of known classes of defense proteins is an important part of the detailed analysis of how plants respond to infection. One of the defense mechanisms used by plants involves antimicrobial peptides (AMPs) that form part of the innate immunity of plants. It is known that these peptides are over-represented in plant genomes and that different classes share structurally similar motifs. Typical defensin structure consists of an α-helix and a triple-stranded, anti-parallel β-sheet. This structure is stabilized by disulfide bonds between cysteine residues, of which there characteristically are eight. These conserved structures are central to their proposed modes of action.

The first grapevine defensin was recently isolated and characterized to be a non-morphogenic plant defensin that inhibits fungal growth through membrane permeabilization (De Beer and Vivier, 2008). Moreover, the peptide has been shown to be expressed only from the onset of berry ripening and expression was non-inducible through wounding, infection of hormone treatments (De Beer and Vivier, 2008). The peptide had strong antifungal activity against *Botrytis cinerea*, *Fusarium*

solani, *F. oxysporum* and *Verticillium dahliae* under *in vitro* conditions. Overexpression of this defensin in transgenic tobacco, however, led to inconclusive results regarding its ability to decrease disease susceptibility against *B. cinerea* in this host, partly due to overlapping peptide activities present in the tobacco host (De Beer, 2008). Infection studies using grapevine pathogens with model plant systems such as tobacco may not yield meaningful results, as these pathogens are unlikely to infect non-host plants.

Following the previous work, purified Vv-AMP1 was evaluated against a panel of grapevine pathogens that could be cultured and sporulated in plate assays to determine the *in vitro* antifungal activity. These pathogens include: *Phomopsis viticola*, which causes cane and leaf spot and infects leaves, shoots and stems; *Phaemoniella chlamydospora*, which is involved in Petri disease (also called Esca, or "black goo" disease) and infects the woody tissue of trunks; *Cylindrocarpon liriodendri*, which causes black foot and infects the roots, crown and rootstock and *Diplodia seriata* which causes "black dead arm" disease, infecting the internal tissues of stems and trunk and may also cause bunch rot.

The peptide proved highly effective at inhibiting growth of all pathogens tested, some at extremely low concentrations (4.6 µg/ml). Moreover, to facilitate *in* vivo analysis of Vv-AMP1 against grapevine pathogens, a putative transgenic population of *V. vinifera* cv. Sultana overexpressing the peptide was obtained and genetically and phenotypically characterized. The transgenic population was challenged with *B. cinerea* in a detached leaf infection assay to evaluate a potential resistance phenotype.

4.3 Materials and Methods

4.3.1 Plant material and microbial strains

A putatively transformed population of *V. vinifera* (cv. Sultana) was obtained from the grapevine transformation and regeneration platform of the IWBT (the transformations were kindly performed by Dr. Krishnan Vasanth). *Agrobacterium tumefaciens* strain EHA 105, transformed with a binary expression vector containing *Vv-AMP1* (constructed by Dr. A. De Beer and reported in De Beer, 2008), was used for the transformation of grapevine as described by Franks et al. (1998). The plant

expression vector drives the constitutive expression of *Vv-AMP1* through the CaMV35S (cauliflower mosaic virus Cabb B-JI isolate 35S) promoter and the OCS (octopine synthase) terminator while the kanamycin resistance gene is under the control of the nopaline synthase gene promoter and terminator (Pnos and Tnos) and provided the antibiotic resistance marker to select transformed cells and putative transformed plantlets from untransformed material. Putative transgenic plants were maintained on Murashige and Skoog basal salt mixture (Murashige and Skoog, 1962) in a growth chamber. A number of plantlets for each individual transgenic line were clonally propagated from the primary transformants, hardened off and maintained in a greenhouse at 25°C and moderate humidity.

All fungal isolates were obtained from Department of Plant Pathology (DPP) Stellenbosch University with the exception of *B. cinerea* which was obtained from the IWBT culture collection. Table 4.1 lists all pathogen strains used in this study as well as specific growth and sporulation conditions for each strain.

Table 4.1 Growth and sporulation media and incubation period to obtain sporulating cultures of grapevine pathogens used in this study. (Culture and sporulation conditions are described as recommended by Dr. Lizel Mostert, Department of Plant Pathology, Stellenbosch University)

Fungal species	**Isolate number**	**Sporulation media**	**Incubation period to obtain sporulating cultures**
Botrytis cinerea	N/A	Potato dextrose agar (PDA)	1 week
Diplodia seriata	STE-U 4440	Oatmeal Agar (OA)	2 – 4 weeks
Phaeomoniella chlamydospora	STE-U 6364	PDA	2 – 4 weeks
Cylindrocarpon liriodendri	STE-U 6171	PDA	2 weeks
Phomopsis viticola	STE-U 5916	PDA/OA	2 – 4 weeks

4.3.2 PCR screen and Southern Blot analysis of transgenic lines transformed with *VvAMP1*

Transgenic grapevine lines transformed with the *VvAMP1* gene were PCR screened and analyzed by Southern Blot to confirm the integration of the transgene and determine the copy number of each line.

For genomic DNA extraction, harvested grapevine leaf tissue was frozen in liquid N_2 and ground to a fine powder. Extractions were performed in accordance with an established protocol (Steenkamp et al., 1994).

To confirm positive integration of the Vv-AMP1 cassette, PCR was performed using the GoTaq system (Promega, Madison, WI, USA). Genomic DNA extracted from transgenic grapevine lines was used as template and together with the primer set Hs-AFP1-5' (5'-GCCGCTCGAGTATTTTTACAACAATTACCAAC-3') and Vitisdef -3' (Vitisdef-3′ (5′-CCGGATCCTTAACAATGCTTAGTGC-3′).

To determine the number of integrated copies of the transgene in each transgenic line, 50 µl of genomic DNA was digested with *Xba*I and *Bgl*II in Buffer 2X Tango™ (Fermentas). The digested DNA was separated on a 1% (w/v) agarose gel and thereafter transferred overnight to a positively charged nylon membrane (Amersham Biosciences, NJ, USA) as described by Sambrook et al. (1989). After transfer, the membrane was UV-cross linked to prevent loss of DNA. The membranes were suitably probed with a DIG-labeled *VvAMP1* probe. Pre-hybridization and hybridization was performed at 37^{o}C using DIG Easy-Hyb granules (Roche Diagnostics GmbH, Mannheim, Germany). Detection was performed with CSPD according to the manufacturer's protocol (Roche Diagnostics GmbH, Mannheim, Germany).

4.3.3 RNA isolation and Northern Blot analysis of transgenic lines transformed with *VvAMP1*

Isolation of RNA was performed according to the protocol of Chang et al. (1993). Total extracted RNA was separated on a 1.2% (w/v) formaldehyde gel. The gel was rinsed in DEPC-treated dH_2O and transferred to positively charged nylon membranes (Sambrook and Fitsch, 1989). The membranes were UV-cross linked where after pre-hybridization and hybridization took place at 50°C. The membranes were suitably probed with a DIG-labeled Vv-AMP1 probe. Detection was performed

with CSPD according to the manufacturer's protocol (Roche Diagnostics GmbH, Mannheim, Germany)

4.3.4 Antimicrobial activity of purified Vv-AMP1 peptide against pathogens

The quantitative antifungal activity of the antifungal peptides was measured using a microspectrophotometric antifungal assay (Broekaert et al., 1990). Purified Vv-AMP1 peptide was obtained from the IWBT; details of the purified peptide were described in De Beer and Vivier (2008). The assay was performed in a 96 well microtiter plate (Nunc, Roskilde, Denmark). Each individual reaction contained 100 µl half strength PDB with 2000 fungal spores and a range of peptide concentrations. The control wells contained no peptide. Reactions were done in triplicate. The plates were incubated in the dark at 25°C for 2 days. Microspectrophotometric readings were taken every 24 hours at 595 nm.

Measurements (A_{595}) were taken at 0 h, 24 h and 48 h using a PowerwaveX microplate reader (Bio-Tek instruments inc.). Values were corrected by subtracting the 0 h value from the 24 h and 48 h measurements. Activity is expressed in terms of the percentage growth inhibition and can be calculated with the formula:

$$\%Inhibition = 100 - \left(\frac{Corrected(A_{595})_{CONTROL} - Corrected(A_{595})_{SAMPLE}}{Corrected(A_{595})_{CONTROL}} \times 100 \right)$$

Microscope images were simultaneously obtained from the antifungal assays with an Olympus IX70 inverted microscope. Images were captured with the Analysis® software (Olympus Soft Imaging Solutions GmbH). The ability of Vv-AMP1 to permeate the fungal membrane was evaluated using a propidium iodide (PI) uptake assay (Gangwar et al., 2006). The permeabilization assay consisted of 200 µl half-strength potato dextrose broth, 2 x 10^4 ml^{-1} fungal spores. Samples were incubated at 25ºC for 48 h. After incubation, the samples were treated with Vv-AMP1 for 15 minutes, then washed with phosphate-buffered saline (PBS) and stained for 10 min in PI staining solution (25 µg/ml PI in PBS). Samples were viewed with an Olympus IX 81 inverted fluorescent microscope. Images were captured using the CelliR® digital camera and software system (Olympus Soft Imaging Solutions GmbH). PI reacts with nucleic acids but is unable to cross the fungal membrane. The

presence of fluorescence is therefore an indication of compromised fungal membranes.

4.3.5 Detached leaf infection assays

VvAMP1 transgenic Sultana lines were challenged with *B. cinerea* through detached leaf infection assays. Leaves were harvested from plants growing under greenhouse conditions. Four individual transgenic lines were chosen at random and three plants from each individual line were tested. Untransformed Sultana plants were used as control plants. Three leaves were harvested from each plant. Leaves chosen were fully developed and expanded. Leaves were placed in sterilized plastic containers on sterilized Whatman paper, moistened slightly with sterilized dH_2O. Containers were closed tightly and leaves were pre-conditioned for 24 h by placing containers in an infection room at a constant temperature of 25°C.

Dry *B. cinerea* spores were hydrated in sterilized dH_2O, 24 h prior to infection. On the day of infection, spores were counted and diluted in 50% grape juice.

Pre-conditioned leaves were inoculated with prepared *B. cinerea* spores by spotting 10 µl of spore suspension onto the surface of the leaf. Each spot contained 2000 spores. Approximately 4-6 spots were made per leaf, depending on leaf size. Infections were followed for six days, both visually and by recording lesion sizes at 24 hour intervals. Differences in lesion sizes were plotted daily and the course of the infection was photographed.

4.3.6 Statistical analysis

A custom program was written in perl to parse the data (Jacobson, 2010), calculate the means and standard deviations and perform an all-against-all set of t-tests to determine if there were significant differences (p value < 0.05) between plant lines at each time point for each of the detached leaf infection assays. A graph was created to represent the statistical relationships amongst the plant lines with an edge created whenever two plant line populations were statistically indistinguishable from one another. The resulting graph was visualized in Cytoscape (Shannon et al., 2003; Cline et al., 2007).

4.4 Results

4.4.1 In vitro analysis of Vv-AMP1 against grapevine pathogens

Purified Vv-AMP1 peptide was tested *in vitro* against a selection of fungal pathogens that cause serious diseases of grapevine. The pathogens selected were those that can be cultured on plates to sporulate and were evaluated for their sensitivity towards Vv-AMP1 using a dose-response growth inhibition assay. Vv-AMP1 showed a marked inhibition of antifungal growth when tested against *Diplodia seriata* (Figure 4.1), *Cylindrocarpon liriodendri* (Figure 4.2), *Phomopsis viticola* (Figure 4.3) and *Phaemoniella chlamydospora* (Figure 4.4). Figures 4.3(B) and 4.4 present the results of assays accurately determining the IC_{50} values of the Vv-AMP1 peptide for *P. viticola* and *P chlamydospora*, respectively. In all assays, the control samples show exponential growth over 48 hours while the assays containing 4.8 µg/ml and 9.6 µg/ml of peptide respectively are severely inhibited after only 24 hours of growth, as well as after 48 hours.

The assays were performed in triplicate and standard deviation was calculated and is represented by error bars.

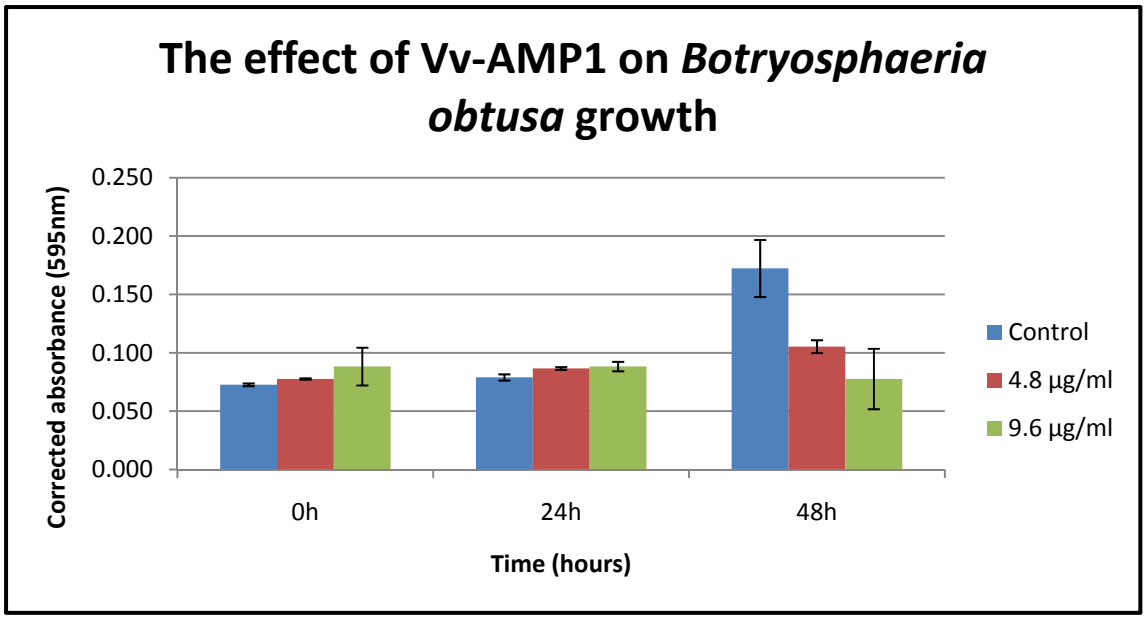


Figure 4.1 Antifungal activity of Vv-AMP1 against *B. obtusa* over a period of 48 hours. Samples treated with 4.8 mg/ml and 9.6 mg/ml respectively both show inhibition of growth when compared to control after 48 hours.

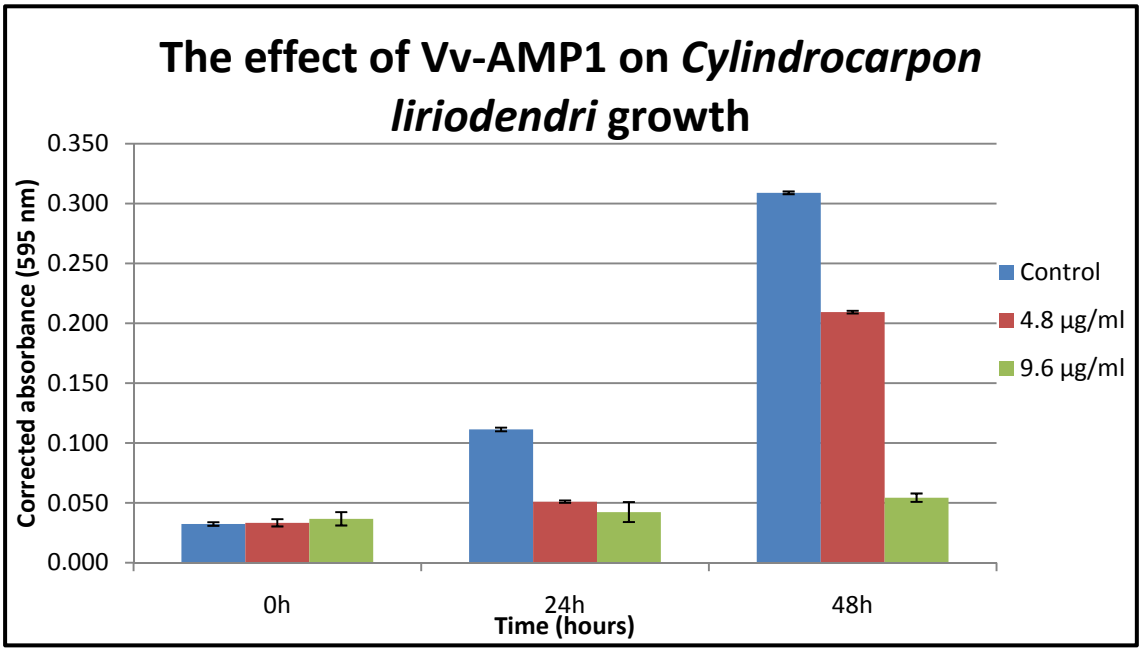
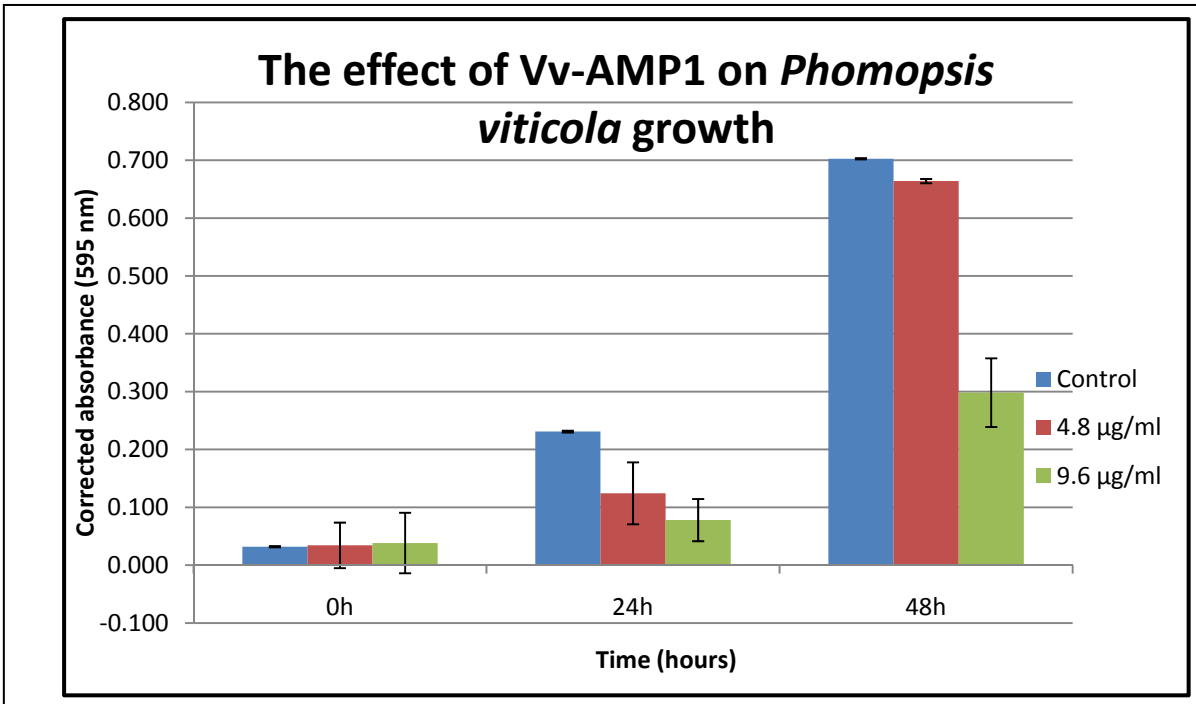
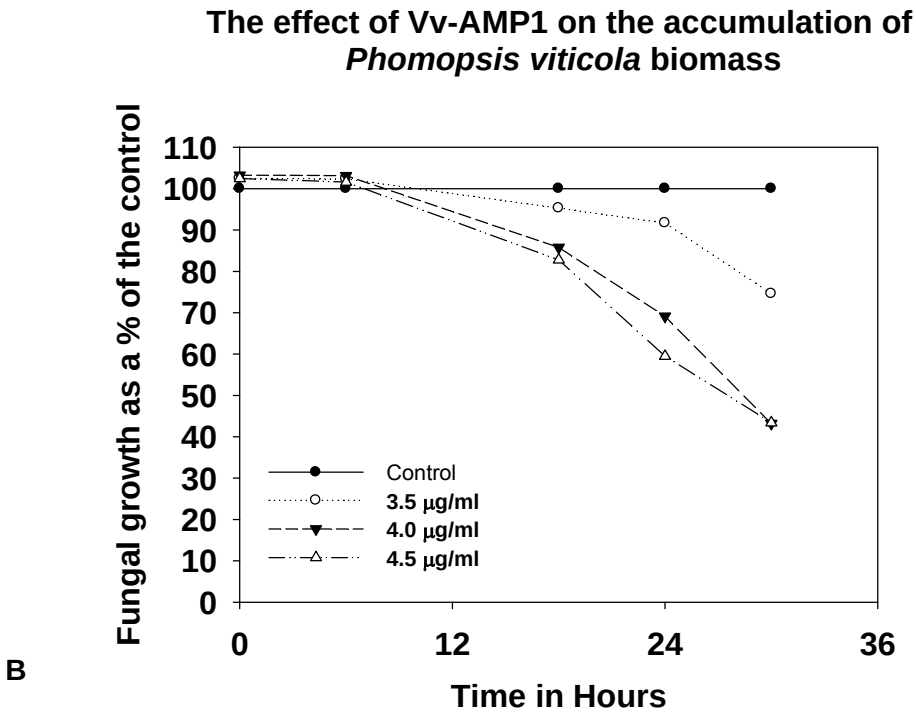


Figure 4.2 Antifungal activity of Vv-AMP1 against *C. liriodendri* over a period of 48 hours. Samples treated with 4.8 mg/ml and 9.6 mg/ml respectively both show inhibition of growth when compared to the control sample after 24 hours. After 48 hours, the sample treated with 4.8 mg/ml of peptide showed moderately inhibited growth and the sample treated with 9.6 mg/ml peptide showed severe inhibition of growth.





B

Figure 4.3 (A) Antifungal activity of Vv-AMP1 against *P. viticola* over a period of 48 hours. Samples treated with 4.8 mg/ml and 9.6 mg/ml respectively both show inhibition of growth when compared to the control sample after 24 hours. After 48 hours, the sample treated with 4.8 mg/ml of peptide showed slightly inhibited growth and the sample treated with 9.6 mg/ml peptide showed severe inhibition of growth. (B) Antifungal activity of Vv-AMP1 against *Phomopsis viticola* expressed as a percentage of growth of the control sample. The concentration of Vv-AMP1 peptide required to inhibit fungal growth by 50% is 4.0 µg/ml.

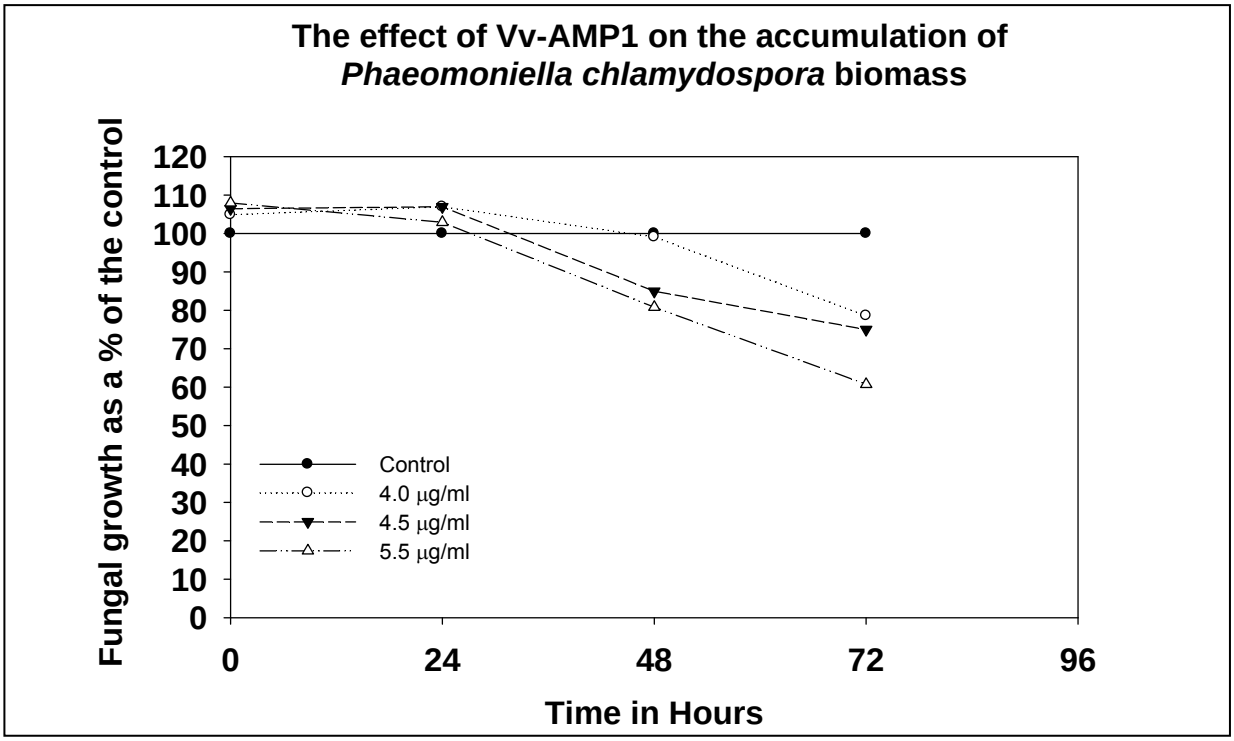


Figure 4.4 Antifungal activity of Vv-AMP1 against *Phaemoniella chlamydospora* expressed as a percentage of growth of the control sample. The concentration of Vv-AMP1 peptide required to inhibit fungal growth by 50% is 5.5 µg/ml.

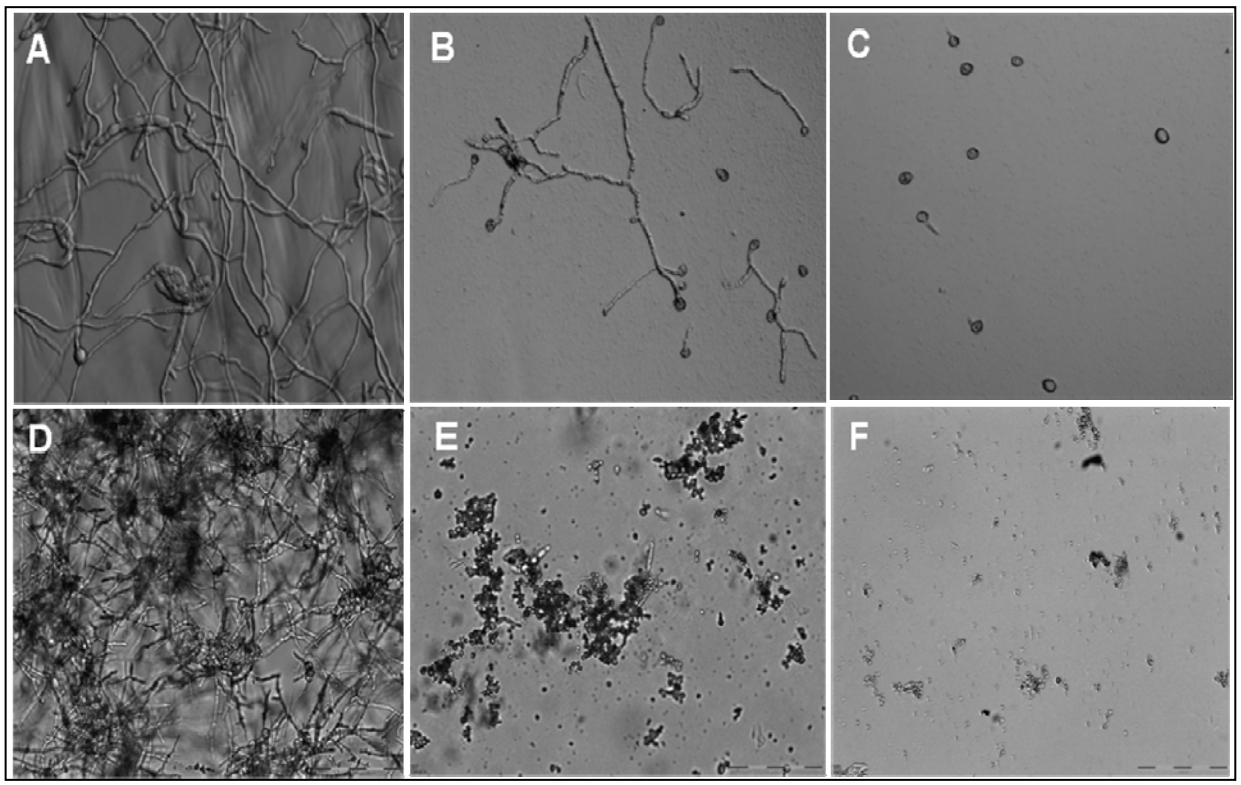


Figure 4.5 (A – F) Light microscope images of *B. cinerea* (A-C) and *P. viticola* (D-F) treated with different amounts of Vv-AMP1 peptide. A and D are the control samples of each pathogen respectively, B is treated with 20 µg/ml, C is treated with 30 µg/ml, E is treated with 4.8 µg/ml and F with 9.6 µg/ml.

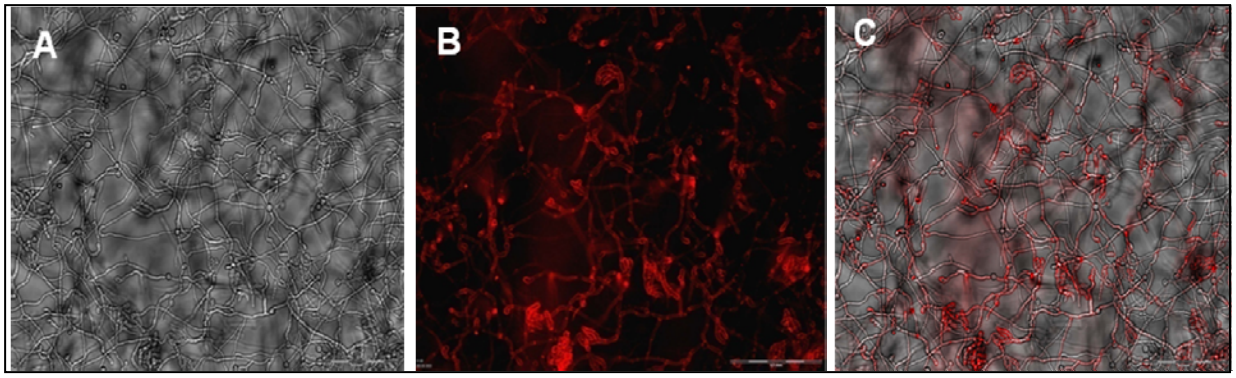


Figure 4.6 (A – C) Microscope images of *P. viticola* cultures grown untreated for 24 hours, then treated with 13 µg/ml Vv-AMP1 and propidium iodide (PI). A is the light microscope image, B is the fluorescent microscope image and C is an overlay of the two images. The fluorescence is an indication of a compromised membrane.

Microscopic analysis of the *in vitro* assays revealed severe effects on fungal growth in samples treated with the purified peptide (Figures 4.5 and 4.6). All treated cultures showed severe reduction of hyphal elongation and biomass development. When present in high concentrations, spore germination was also inhibited by the presence of the peptide (Figure 4.5 C and F). This was observed in two of the pathogens, *B. cinerea* and *P. viticola*. No microscopic analysis of the other pathogens was performed. *P. viticola* proved a great deal more sensitive to the peptide than *B. cinerea*, being inhibited at much lower concentrations of Vv-AMP1. Figure 4.6 shows a propidium iodide (PI) uptake assay where *P. viticola* was first grown for 24 hours without the presence of the peptide. Shortly before the images were taken, the samples were treated with Vv-AMP1 and subsequently stained with PI. The visible fluorescence seen in the treated samples indicated that the fungal membranes were severely compromised within 15 minutes after treatment with the Vv-AMP1 peptide.

Microscopic analysis also confirmed the previous observation by De Beer and Vivier (2008) that Vv-AMP1 does not cause hyperbranching in treated cultures.

4.4.2 Analysis of a transgenic grapevine population overexpressing Vv-AMP1

Twenty five putative transgenic lines of *V. vinifera* (cv. Sultana) transformed with the *Vv-AMP1* expression cassette was kindly obtained from Dr. Krishnan Vasanth from the IWBT transformation and regeneration facility and subjected to genetic and phenotypical analysis. The genetic analysis included PCR, Southern and northern blot analysis (Figure 4.7 and Table 4.2). PCR screening confirmed the presence of

the transgene in all lines of the putative transgenic population. Untransformed *V. vinifera* material was used as control. No amplified product is detected in the control lines as the primers are designed to amplify a copy of the transgene which includes a part of the expression cassette, preventing amplification of the native gene (Figure 4.7 A). Southern blot analysis revealed nine individually transformed transgenic lines, as seen by the individual banding patterns. Untransformed lines indicated two native copies of the *Vv-AMP1* gene and transgenic lines showed multiple copies, with each band representing an integration of the *Vv-AMP1* transgene (Figure 4.7 B). All nine individual transgenic lines tested northern blot positive, confirming expression of the *Vv-AMP1* gene. The native gene is not expected to be expressed in untransformed lines since the native *Vv-AMP1* gene is only expressed in berry tissue at the onset of berry ripening, and not in leaves. Any expression detected in Northern blot analysis thus indicates the expression of the transgene (Figure 4.7 C). Figure 4.7 (A - C) shows examples of PCR screens, Southern and northern blot data whereas Table 4.2 summarizes the results of the molecular characterization of the transgenic *V. vinifera* lines overexpressing *Vv-AMP1*.

Table 4.2 Summary of molecular characterization of nine transgenic *V. vinifera* (cv. Sultana) lines overexpressing the Vv-AMP1 plant defensin. Characterization included PCR screening, Southern and Northern blot analysis. Number of integrations refers to the number of copies of the *VvAMP1* transgene integrated into the genome. A "+" denotes a positive result and a "-" a negative result. WT indicates the untransformed *V. vinifera* cv. Sultana lines used as control.

Plant line	WT	6	7	8	9	10	14	17	18	19
PCR	-	+	+	+	+	+	+	+	+	+
Southern Blot	+	+	+	+	+	+	+	+	+	+
Northern Blot	-	+	+	+	+	+	+	+	+	+
Number of transgene Integrations	0	2	6	2	3	2	3	2	2	5

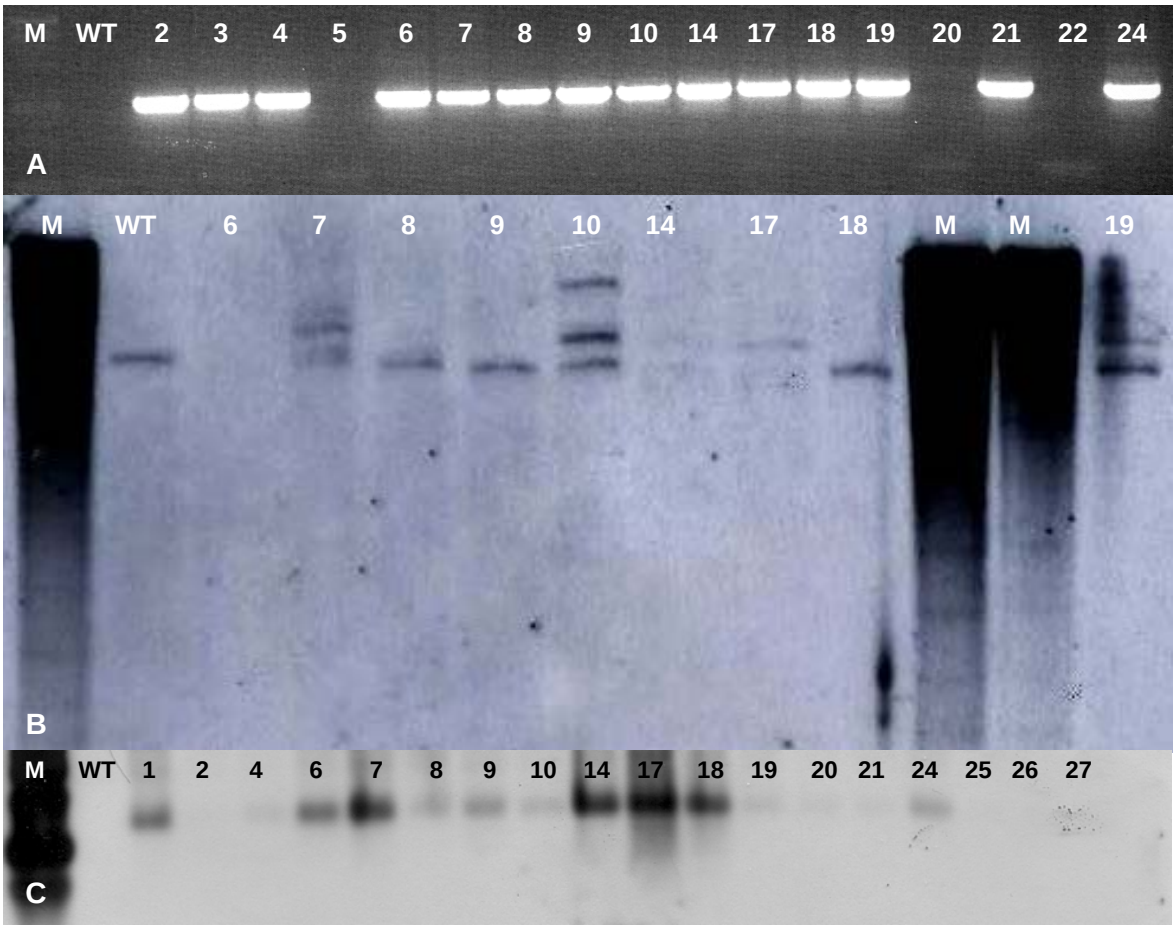


Figure 4.7 (A - C) Representative examples of PCR screen (A), Southern (B) and Northern (C) blots indicating integration and expression of *Vv-AMP1* in the transgenic *V. vinifera* lines. Lanes marked M denote molecular markers (Fermentas Lambda Pst*I* marker). WT denotes the untransformed control lines and numbered lanes indicate putative transgenic lines.

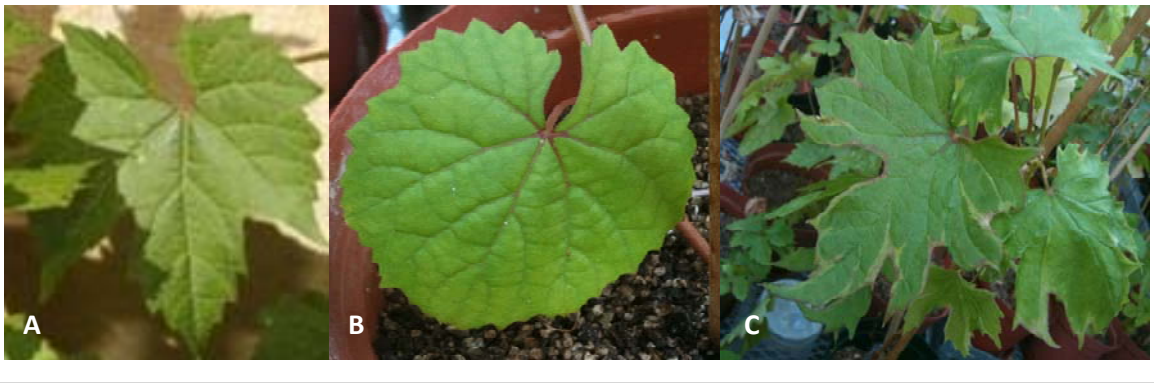


Figure 4.8 (A-C) Normal growth phenotype (A) compared to abnormal growth phenotypes observed in transgenic *Vv-AMP1* lines 19 (B) and 17 (C). Leaves showed unusual growth and formation when compared to typical *V. vinifera* cv. Sultana development.

Lines that were confirmed to be independent transgenic lines with transgene expression were multiplied and hardened off. Phenotypically, most of the lines appeared similar to the control in terms of growth speed, plant size and general appearance of the vegetative organs, but some lines (lines 19 and 17) showed abnormal leaf morphology as well as altered internode/node arrangements (Figure 4.8). These growth phenotypes showed no correlation to number of integration events. A subset of lines were randomly selected for infection with *B. cinerea* to investigate the *in planta* defense response of the overexpressed lines. Of the grapevine pathogens tested *in vitro*, *B. cinerea* was not the most sensitive organism to the Vv-AMP1 peptide, but it provides a convenient pathosystem in grapevine as it infects leaves, whereas the other selected pathogens typically infect woody stems and established leaf infection systems are not available.

For this first screen, a detached leaf assay was used where three fully expanded leaves of three clonal copies of lines 6, 14, 18 and 19 were infected with four to six spots per leaf. *B. cinerea* rapidly infected both transgenic and untransformed leaves and primary lesions were visible on all leaves after 2 days of incubation at 25°C and 100% humidity. Spreading lesions developed after that and lesion sizes (diameter in mm) were measured daily for six days. After day 5, accurate measurements became difficult as lesions tended to flow into one another. The overall infection rate was 95%.

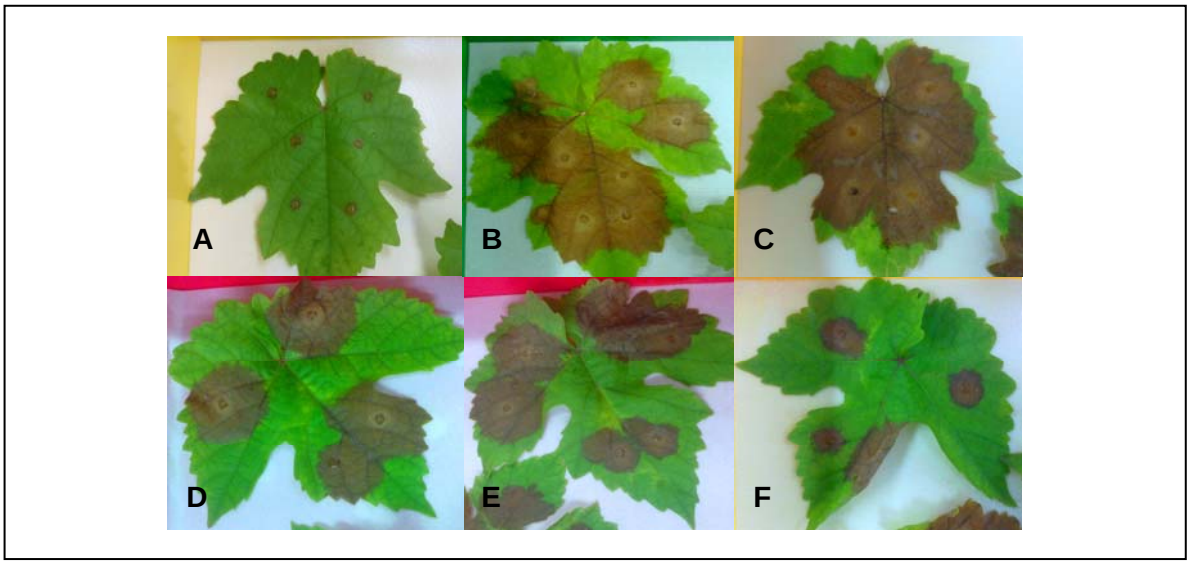


Figure 4.9 (A-E) Lesion formation on detached grapevine leaves, six days after infection with *B. cinerea*. Panel A shows lesion formation on an untransformed control leaf, two days after infection. Panel B shows untransformed *V. vinifera* cv. Sultana line used as the control. Six days after infection, the control leaf is mostly colonized by the pathogen. Panels C to F represent transgenic *Vv-AMP1* lines 6, 14, 18 and 19, respectively. Lines 6 and 19 visually appear to show a higher degree of resistance to infection with slightly smaller lesions when compared to the control line and transgenic lines 14 and 19.

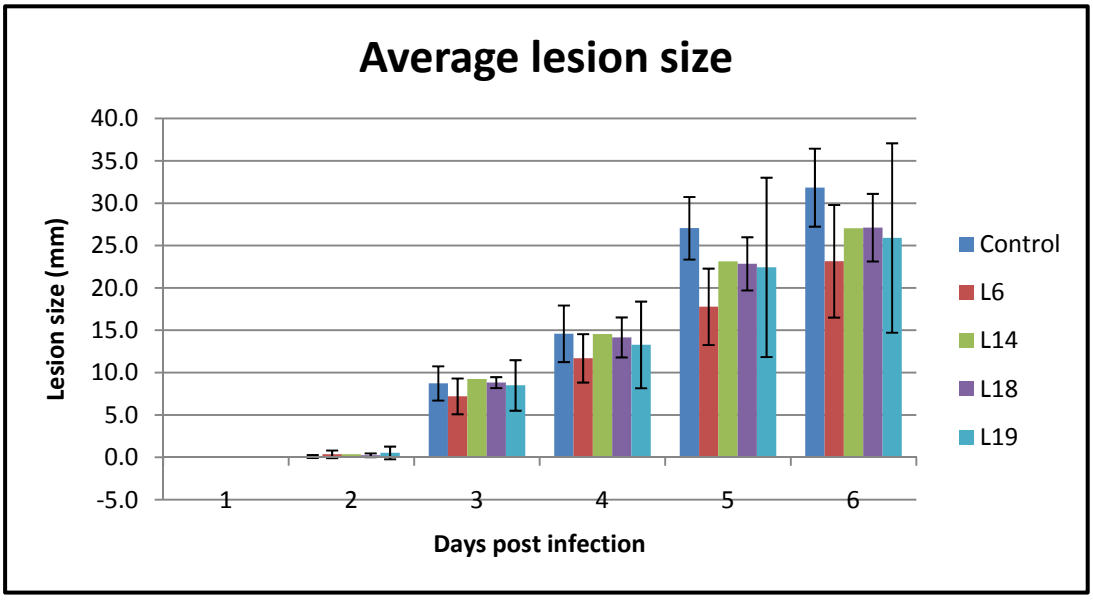


Figure 4.10 Measurements of average lesion size of detached leaves infected of both transgenic *Vv-AMP1* grapevine lines and untransformed control lines with *B. cinerea*, six days after inoculation and growth at 25°C and 100% humidity. The graph indicates the average lesion size of the over a period of six days. Error bars denote the standard deviation of the data analyzed. After six days, transgenic lines 6 and 19 suggest a marginally higher degree of resistance when compared to the control line.

Typical lesion appearance at the end of the experiment is shown in Figure 4.9 and the average lesion sizes over the infection period are shown in Figure 4.10. From this detached leaf assay against *B. cinerea*, it is observed that the overexpression leads to a mild resistance phenotype when compared to the control. Statistical analysis of the lesion measurements, where a t-test was used to evaluate and compare all lesions against one another, indicated that the transgenic population could be separated from the untransformed control based on lesion sizes (Figure 4.11).

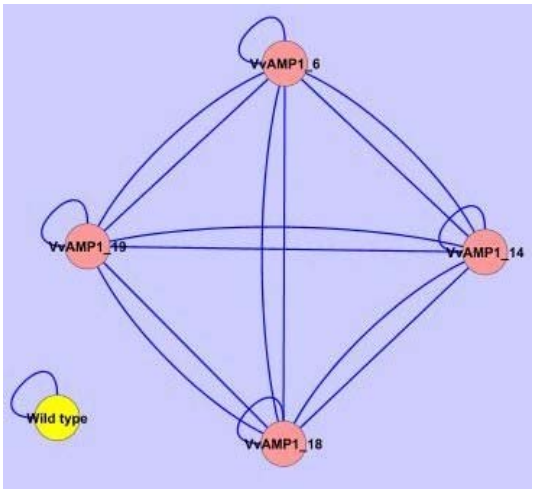


Figure 4.11 Visual representation of statistical analysis (generated through Cytoscape) signifying the statistical relationships between untransformed control lines ("Wild type") and transgenic *Vv-AMP1* (VvAMP1_6, VvAMP1_14, VvAMP1_18 and VvAMP1_19) plant lines. A blue line denotes the statistical relationship between plant lines. An edge is created whenever two plant lines are statistically indistinguishable from one another. The graph indicates while all four transgenic *Vv-AMP1* lines are indistinguishable from one another, the control line is distinguishable from the transgenic lines. This significant difference suggests a higher degree of resistance of the transgenic lines when compared to the untransformed controls.

4.5 Discussion

The focus on increasing the disease resistance of crops to pathogen attack through recombinant DNA technology has been increasing in recent years. A number of such recombinant resistance studies on a variety of crop plants transformed with genes encoding for antifungal peptides have been completed (Aerts et al., 2007; Swathi Anuradha et al., 2008; Dai and Beachy, 2009; Zainal et al., 2009). These studies indicate that the introduction and overexpression of genes encoding for antifungal peptides have an effect in enhancing the plant's natural defense against pathogen infection and disease. The active production of these antifungal peptides by the plant seems to provide the plant with the ability to inhibit fungal growth and slow down infection.

The grapevine defensin Vv-AMP1 was highly effective against grapevine pathogens when used in *in vitro* antifungal assays. Not only does the peptide inhibit the growth (and, at very high concentrations, even germination) of the pathogens, it does this at remarkably low concentrations (as low as 4.0 µg/ml). This high level of activity, even at extremely low doses, makes it a peptide of interest for *in planta* disease resistance studies, the hypothesis being that if the plant expresses even low levels of peptide in the tissue, the presence of such a highly active peptide could significantly decrease the plant's susceptibility to fungal infection. The microscopic analysis also confirmed the peptide to be non-morphogenic with a very strong membrane disruption activity (see Figure 4.5 and 4.6).

Propidium iodide (PI) uptake assays showed clear fluorescence in the presence of Vv-AMP1. PI is incapable of crossing the intact microbial membranes of living cells and only fluoresces in the presence of nucleic acids. The fluorescence observed is therefore an indication of a compromised membrane. This supports the theory that the mode of action of the Vv-AMP1 peptide is associated with membrane permeabilization. Recent evidence has proposed the membrane disruption activity of the defensins to be a secondary response (Amien et al., 2010; van der Weerden et al., 2010). Moreover, these studies propose that an intact cell wall may be required for this specific mode of action (López-García et al., 2010). These studies suggest that the cell wall itself might play a role in the mode of action of defensins, possibly through interactions with proteinaceous receptors located in the cell wall (Amien et al., 2010).

However, in our PI assays, membrane disruption appeared very quickly after treatment of hyphal culture with the Vv-AMP1 peptide. This indicates a strong membrane permeabilization interaction. It would be of interest to perform experiments to evaluate whether this response is dependent on other factors and whether it is indeed a secondary response rather than the primary mode of action.

The possibility to evaluate the effectiveness of the Vv-AMP1 peptide against grapevine pathogens in *in vitro* assays are limited to pathogens that can be cultured on plates to form sporulating cultures. Many grapevine pathogens which cause economically damaging crop losses are not culturable and can therefore only be evaluated in an *in planta* environment. Previous overexpression analysis of this peptide in tobacco as a model plant (De Beer, 2008) is not useful in this regard, since many of these pathogens do not infect tobacco. To this end, a transgenic population of grapevine overexpressing the Vv-AMP1 peptide was obtained and genetically characterized. Nine independently transformed lines with confirmed transgene expression could be identified in this study. These lines are important resources to further characterize the *in vivo* role of the Vv-AMP1 defensin against grapevine pathogens. Individual plants from transgenic lines 17 and 19 showed abnormal growth phenotypes, with atypical leaf formation when compared to untransformed Sultana plants. Abnormalities were absent in most of the transgenic population, and atypical leaves were not used for infections. The uncharacteristic growth patterns could possibly be attributed to the effect of transgene integration patterns into the genome.

Western blot analysis on these lines proved inconclusive (results not shown). The protein extraction methods tested were inefficient to isolate the smaller peptide fractions from leaf tissue. Furthermore, the compounds within the extraction buffers interfered with the Western blot analysis itself (data not shown). These extractions and analysis needs to be optimized and repeated to accurately determine the presence of the Vv-AMP1 peptide in leaves, stems or roots. Since the peptide presence could not be confirmed in the *in planta* environment with Western blot analysis, the presence were indirectly tested by evaluating the peptide activity in infection studies. The antifungal activity of the overexpressed peptide was evaluated through detached leaf infection assays against *Botrytis*. *B. cinerea* was chosen as a first screening organism, given its known ability to form lesions on infected leaves.

Comparison of transgenic lines with control lines after infection with *B. cinerea*, both in terms of lesion type and average lesion size that develop over a six day period, suggest that the transgenic lines have a marginally higher degree of resistance than the control. Statistical analysis of lesion measurements (incorporating means, standard deviations and t-tests) indicated that while all four transgenic lines tested were statistically indistinguishable from one another (and thus behaved as a population), they were all distinguishable from the control lines.

The low level of improved disease resistance could possibly be attributed to various factors. *B. cinerea* is one of the more resistant pathogens tested against Vv-AMP1, with IC_{50} values of 13 µg/ml. If Vv-AMP1 is present in the leaves of transgenic grapevine at relatively low levels, it will affect the resistance phenotype.

Moreover, the detached leaf assay also activates a wound response prior to infection and this background defense response might mask the defense phenotype linked to the overexpression of Vv-AMP1. The infection assay should be followed by a whole plant infection assay to follow the defense phenotype against *B. cinerea* and other grapevine pathogens such as powdery and downy mildew without the interference of the wound response. The infection method should also be further optimized with regards to leaf age, position and developmental stage since these factors could also influence the susceptibility of the plant to the pathogen. Also, *B. cinerea* is known to secrete a number of substances during colonization and infection of a plant. These substances (such as cutinases, pectinases and proteases) may alter the apoplastic environment and interfere with the ability of the antimicrobial peptide to inhibit the fungal growth. Proteases secreted by the pathogen may serve to degrade defense proteins and peptides present in the apoplast (Prins et al., 2000).

In conclusion, *in vitro* studies provided evidence that the Vv-AMP1 defensin is strongly active against grapevine pathogens. This promising result, in combination with a confirmed transgenic population of grapevine overexpressing the peptide, provides significant scope to evaluate the *in vivo* functions of the peptide in defense against grapevine pathogens. Furthermore, the small but significant increase in disease resistance obtained in the detached leaf infection with *B. cinerea* confirmed the peptide to be produced and active in the lines. The next step would be to challenge these lines with more sensitive grapevine pathogens in whole plant infection assays, as well as assays on stems for stem specific pathogens.

Many studies have shown that the targeted overexpression of antifungal peptides could lead to a stable transgenic plant with improved resistance against plant pathogens (Dai and Beachy, 2009; Zainal et al., 2009). Transgenic studies in grapevines overexpressing antimicrobial peptides (eg. Magainan-2) have shown promising results towards improved resistance and symptom reduction when challenged with pathogens such as *A. vitis*, *B. cinerea*, *Erysiphe necator* and *U. necator*. (Vidal et al., 2006; Rosenfield et al., 2010), but to date no strong, definite resistance has been established. The results obtained in this study warrants further investigation into the potential disease resistance of transgenic *Vv-AMP1* grapevine, specifically against a wider range of grapevine pathogens.

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Chapter 5

General discussion and conclusion

GENERAL DISCUSSION AND CONCLUSION

5.1 General discussion and conclusion

The principle challenge facing modern agriculture in recent years has unquestionably been achieving and maintaining sustainable agriculture through increasing and improving crop yields and quality while simultaneously lowering production costs. The world's rapidly growing population and changes in climate and environmental conditions have only added to the urgency of finding solutions to address these challenges. One of the key problems experienced by modern agriculture is the efficient management of crop pests and diseases (Walker, 1983; Shah, 1997). Crop losses attributed to pest and disease annually is significant and preventing or reducing such losses would be of great fiscal value, specifically for economically important food crops, such as corn, soy, rice and grapevine.

While disease management of crops has in the past depended heavily upon the use of regular chemical treatments, recent concerns have been raised regarding the long-term effects of pesticides and fungicides on both the environment and human health. The excessive use of chemicals has also led to resistant pathogen strains, which are no longer affected by fungicide treatment. These issues have required research to investigate safer, more sustainable alternatives to combat the occurrence and spread of disease (Lewis and Papavizas, 1991; Harms, 1992).

In this search for safer, more effective solutions to the problem researchers have begun to turn to the plant's natural ability to defend itself. Through exploring and understanding the natural defense mechanisms of plants (including antimicrobial peptides and plant defensins) strategies can be developed to harness these naturally developed systems. Through genetic engineering approaches even susceptible crops can be protected against disease (Montesinos, 2007; Swathi Anuradha et al., 2008; Dai and Beachy, 2009).

The discovery of the first grapevine defensin, Vv-AMP1, has created an opportunity to investigate the potential of this novel peptide as a tool to explore and improve the natural ability of grapevine to defend itself against pathogen attack.

The *Vv-AMP1* gene was isolated from *Vitis vinifera* and showed high sequence homology to plant defensins. Extensive work done by De Beer and Vivier (2008) to characterize the peptide found it to be highly developmentally regulated, being

expressed only in the berry and only at the onset of berry ripening. Expression could not be induced through wounding, infection with a pathogen or hormone treatment (De Beer and Vivier, 2008).

Investigation into the signal peptide showed that Vv-AMP1 accumulated in the apoplastic region. Recombinantly produced Vv-AMP1 was further characterized and found to have a molecular mass of 5.495 kDa as determined by mass spectrometry. The peptide proved extremely heat stable and showed antifungal activity against most pathogenic fungi tested, specifically against *Fusarium oxysporum, F. solani, Verticillium dahliae* and *Botrytis cinerea* (De Beer and Vivier, 2008). *Vv-AMP1* was overexpressed in tobacco, but infection assays failed to conclude that the presence of the peptide caused a decreased susceptibility of the host plant to pathogen infection when tested against *B. cinerea* (De Beer, 2008).

The scope of this project was to further investigate the potential of *Vv-AMP1* and supplement existing information gained from the work of De Beer and Vivier (2008). This included evaluating and characterizing the antifungal activity of the peptide against a panel of grapevine-specific pathogens as well as studying the effect of peptide overexpression of the peptide within its native host, *V. vinifera*. The results of this study furthered our knowledge of the Vv-AMP1 grapevine defensin and yielded valuable resources for subsequent studies into grapevine disease resistance. This is the first report of a grapevine defensin functionally analyzed in grapevine and one of very few studies of defensins within this host.

The objectives of this study (as outlined in Chapter 1) have been met and the major findings of the study can be summarized as follows:

The promising results obtained from initial *in vitro* antifungal assays (De Beer and Vivier, 2008) prompted further investigation of the antifungal activity of the peptide, specifically against a panel of grapevine-specific pathogenic fungi. For *in vitro* testing, the peptide was required in pure, biologically active form.

Subsequent efforts to recombinantly produce Vv-AMP1 in *Escherichia coli* using the pGEX-2T system proved problematic, prompting investigation and optimization of the production protocol. A number of steps in the production and purification protocol were targeted for improvement, including parameters surrounding the induction conditions as well as multiple steps throughout the purification procedure.

Despite successfully improving the production purification protocol and increasing yield nearly two-fold, the resultant peptide remained biologically inactive and unstable. This is uncharacteristic of the peptide as described by De Beer and Vivier (2008). Both the lack of activity and the observed peptide instability suggests that the produced peptide may not be structurally correct. This could be resultant of incorrect folding after cleaving the peptide from the GST-tag. This may be ascribed to an absence of essential co-factors or molecular chaperones that are not found in the intracellular environment of *E*. *coli*. Another feasible explanation is the occurrence of codon bias, inhibiting the organism's ability to produce the desired peptide with the correct amino acid sequence. Incorrect folding may alter the structure of the peptide, in turn affecting activity and stability.

Though attempts to recombinantly produce Vv-AMP1 failed to produce active peptide, a sample of biologically active Vv-AMP1 (obtained from Dr. A. De Beer, Institute for Wine Biotechnology, Stellenbosch University) was available for *in vitro* antifungal assays.

Vv-AMP1 was tested against a panel of grapevine-specific pathogenic fungi, chosen based on their ability to be cultured and sporulate on plates containing appropriate growth media. Since a number of the most economically damaging grapevine pathogens (such as powdery and downy mildew) are not culturable, they can only be tested in an *in planta* environment and as a result were not suitable for the purposes of this study.

Results of the antifungal assays indicated that the Vv-AMP1 peptide was highly active against all the grapevine pathogens tested. Propidium iodide (PI) uptake assays were used to evaluate the mode of action of the Vv-AMP1 peptide. Membrane disruption appeared rapidly after treatment of the fungal culture with Vv-AMP1 (within 15 minutes). This correlates with the suggested hypothesis that the Vv-AMP1 peptide acts through membrane permeabilization. While recent studies (Amien et al., 2010; van der Weerden et al., 2010) suggest that the membrane disruption activity of defensins may be a delayed secondary response and that intact cell walls may be required for this specific mode of action, the rapid effect of Vv-AMP1 on the fungal membrane indicates a strong and almost instantaneous membrane permeabilization effect.

For *in planta* evaluation of *Vv-AMP1*, a population of transgenic *V. vinifera* (cv. Sultana) lines were obtained and genetically analyzed. These lines had been

transformed with a gene expression cassette containing the *Vv-AMP1* gene and a constitutive promoter, ensuring constitutive expression in all tissues. The transgenic population was genetically characterized to confirm the presence, integration and expression of the *Vv-AMP1* transgene. Nine independently transformed lines with confirmed expression of the transgene were identified. Attempts to confirm the presence of Vv-AMP1 peptide in leaf, root and stem tissue were unsuccessful. As Western blot analysis remained inconclusive, the presence and antifungal activity of Vv-AMP1 in leaf tissue was directly evaluated through detached leaf infection assays.

Despite the fact that *B. cinerea* was determined to be slightly more resistant to growth inhibition by Vv-AMP1 than other grapevine pathogens tested, it's known ability to infect grapevine leaves and form lesions favored it as a first screening organism.

Transgenic and control lines were infected with *B. cinerea* and after six days, a comparison of the transgenic lines with the control lines demonstrated that the transgenic lines performed marginally better than the untransformed controls. Further statistical analysis of the infection data revealed that the transgenic lines were statistically indistinguishable from one another and thus behaved as a population, and also that the transgenic lines were all statistically discernible from the control lines. This confirms that Vv-AMP1 is both present and active in the transgenic lines and that a resistance phenotype can be linked to the overexpression of Vv-AMP1 in the transgenic lines.

Though enhanced resistance against *B. cinerea* is evident in the transgenic population, the level of improved resistance is still low. This marginal improvement in resistance may be ascribed to a number of contributing factors. *B. cinerea* is known to be slightly more resistant to Vv-AMP1 than other grapevine pathogens tested, with an established IC_{50} value of 13 µg/ml (De Beer and Vivier, 2008). If Vv-AMP1 is produced in the leaves of transgenic plants at levels that are relatively low, this could negatively affect the resistance phenotype. In addition, the harvesting of the leaves for the attached leaf assay induces a wound response, which in itself may inhibit or interfere with the effect of Vv-AMP1, thereby masking the defense phenotype linked to Vv-AMP1 overexpression.

Also, the method of infection may need optimization with regards to leaf age, position and developmental stage, as these factors could all influence the

susceptibility of the plant to pathogen attack. *B. cinerea* has also been known to secrete a number of substances during infection of plant tissue. These substances (specifically proteases) may degrade the Vv-AMP1 peptide within the apoplastic environment, thereby interfering with the peptide's ability to inhibit fungal growth.

In conclusion, the availability of recombinantly produced and purified Vv-AMP1 was necessary to facilitate *in vitro* antifungal assays against fungal pathogens. These *in vitro* studies revealed that Vv-AMP1 is highly active against grapevine-specific pathogens. The establishment of a confirmed transgenic population overexpressing Vv-AMP1 increases the scope of evaluating the *in vivo* function of the peptide in grapevine, specifically in defense against infection from grapevine pathogens. Confirmation that the transgenic population showed an improved level of resistance against *B. cinerea* infection warrants further testing of the transgenic population against pathogens that are more sensitive to Vv-AMP1 (e.g. *P. viticola*) and those that can only be tested in *in planta* assays (e.g. downy and powdery mildew). Subsequent research and future work may ultimately lead to a stable transgenic grapevine with a significantly improved resistance against grapevine pathogens.

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